Antiretroviral treatment of HIV/AIDS in rural Tanzania:

Long-term outcome and simplified monitoring tools

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Summary

The scale-up of antiretroviral treatment (ART) of HIV/AIDS in resource-limited settings has been one of the largest public health operations of our time, and by the end of 2009 more than 5 million people were receiving ART in low- and middle-income countries. Although several studies on ART efficacy in Africa have been published, the majority have been carried out in larger cities and usually with short follow-up time. To date, there is a paucity of research from rural settings, which often face additional challenges, such as shortages of health workers, transport difficulties and other logistical constraints. In the first part of the present study we reported long-term clinical and virological outcomes of ART in a rural hospital in Tanzania.

In the second part of this study we addressed the lack of field-friendly tools for virological monitoring of patients on ART. Viral load and resistance testing, as recommended in high-income countries, are rarely available in resource-limited settings due to high costs and stringent requirements for storage and transport of plasma. Consequently, treatment failure in such settings usually passes unnoticed until patients develop severe immunodeficiency, at which stage widespread resistance is likely. Dried blood spots (DBS) are easy to collect and store, and can be a convenient alternative to plasma. Unlike plasma, DBS can be stored and shipped at ambient temperature, thus avoiding the need for cold chain and speedy transport to the laboratory. In our study, under field conditions in rural Tanzania, we assessed the performance of DBS in virological monitoring of patients on ART.

Haydom Lutheran Hospital has provided ART to HIV-infected patients since 2003. A combination of stavudine or zidovudine with lamivudine and either nevirapine or efavirenz is the standard first-line regimen, in accordance with WHO and Tanzanian guidelines. All patients who started ART from October 2003 through December 2007 were included in a longitudinal cohort study. Standard techniques of survival analysis were used to estimate mortality and identify predictors of mortality. Virological efficacy and emergence of drug resistance was assessed in patients who had completed at least 6 months of first-line ART. Viral load and resistance results obtained with a standard plasma-based method were compared with results obtained with the use of DBS.

Paper I and II were epidemiological studies. We found a high mortality in this cohort, particularly the first months after initiating ART: estimated mortality was 19.2%, 29.0% and 41.7% after 3, 12 and 36 months, respectively. Anemia, malnutrition and thrombocytopenia were strong and independent predictors of mortality. A prognostic model based on hemoglobin level appeared to be a useful tool for initial risk assessment: estimated one year mortality was 3.7% in patients without anemia compared to 55.2% in those with severe anemia (<8 g/dL). Among patients who survived the first 6 months and remained in care, however, we found good long-term virological efficacy: viral suppression (<400 copies/mL) was observed in 187 of 212 (88.2%) patients after a median follow-up time of 22.3 months. In total, 18 patients harbored at least one clinically significant drug-resistance mutation, of whom 5 had thymidine analogue mutations associated with broad cross-resistance to nucleoside reverse transcriptase

inhibitors. Although the overall prevalence of drug resistance was relatively low, it increased with time and reached approximately 15% after 3-4 years on ART.

Paper III and IV were laboratory studies. First, we compared the viral load levels in 98 plasma-DBS pairs from patients on ART. In a linear regression model there was a strong correlation, with an R^2 value of 0.75, between the two specimen types. Viral loads were on average slightly higher in plasma than DBS, but the mean difference was only 0.04 $\log_{10}$ copies/mL. However, DBS had reduced sensitivity to detect HIV-1 RNA in samples with low-level viraemia (<3000 copies/mL). Subsequently, we compared genotypic resistance results from DBS with those of plasma in 36 ART-experienced individuals with treatment failure (viral load >1000 copies/mL). Overall, 34 of 36 (94%) DBS specimens were successfully genotyped, and there was high concordance between mutations found in plasma and DBS. Thirty of 34 (88%) patients had identical resistance profiles to antiretroviral drugs in plasma and DBS.

In conclusion, we found a high early mortality in this cohort. Simple laboratory markers, especially hemoglobin level, appeared to be useful for initial risk assessment, and can be of particular use in settings without access to CD4 cell counts. Long-term virological efficacy rates were favorable, and drug resistance appeared to develop at the same rate as in high-income countries. Finally, we found that the use of DBS was a feasible and reliable option for viral load and resistance testing, and we believe that DBS could simplify virological monitoring in resource-limited settings.

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Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral Treatment
BMI	Body Mass Index
CI	Confidence Interval
DBS	Dried Blood Spots
DNA	Deoxyribonucleic Acid
HIV	Human Immunodeficiency Virus
IQR	Interquartile Range
NASBA	Nucleic Acid Sequence-Based Amplification
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT	Reverse Transcriptase
SD	Standard Deviation
TAM	Thymidine Analogue Mutation
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organization

List of papers

I. Johannessen A, Naman E, Ngowi BJ, Sandvik L, Matee MI, Aglen HE, Gundersen SG, Bruun JN. Predictors of mortality in HIV-infected patients starting antiretroviral therapy in a rural hospital in Tanzania. *BMC Infectious Diseases* 2008, 8: 52.

II. Johannessen A, Naman E, Kivuyo SL, Kasubi MJ, Holberg-Petersen M, Matee MI, Gundersen SG, Bruun JN. Virological efficacy and emergence of drug resistance in adults on antiretroviral treatment in rural Tanzania. *BMC Infectious Diseases* 2009, 9: 108.

III. Johannessen A, Garrido C, Zahonero N, Sandvik L, Naman E, Kiyuyo SL, Kasubi MJ, Gundersen SG, Bruun JN, de Mendoza C. Dried blood spots perform well in viral load monitoring of patients who receive antiretroviral treatment in rural Tanzania. *Clinical Infectious Diseases* 2009, 49: 976-981.

IV. Johannessen A, Holberg-Petersen M, Lövgaarden G, Naman E, Ormaasen V, Matee MI, Gundersen SG, Bruun JN. HIV-1 drug resistance testing on dried blood spots is feasible and reliable in patients who fail antiretroviral therapy in rural Tanzania. *Antiviral Therapy* 2010, 15: 1003-1009.

1. Background

1.1. History of HIV/AIDS

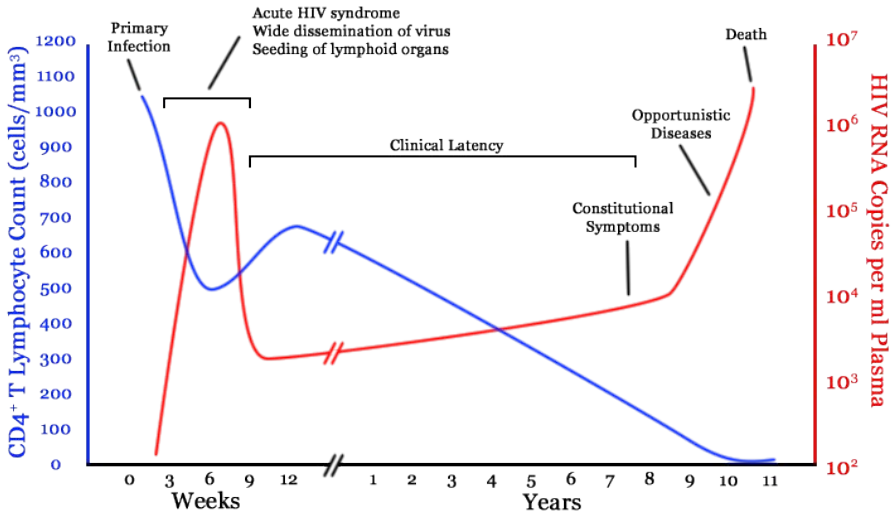
The Acquired Immune Deficiency Syndrome (AIDS) was first described among homosexual men in USA in 1981 [1-5]. Two years later a retrovirus, later known as Human Immunodeficiency Virus (HIV), was identified as the causative agent of AIDS [6-8]. Since then HIV/AIDS has been reported worldwide, and more than 25 million individuals have died from AIDS over the past three decades [9].

HIV invades CD4 positive cells, and shortly after onset of the primary infection there is a significant decline in the CD4 cell count followed by a partial recovery [10]. Thereafter, the CD4 cell count decreases gradually over a period that usually lasts for several years, during which most patients are asymptomatic and many still unaware of their HIV infection. When the CD4 cell count has dropped to a critical level, usually below 200 cells/μL, cellular immunodeficiency renders the patients susceptible to opportunistic infections and malignancies [11]. Figure 1 gives a schematic overview of disease progression in relation to CD4 cell count and viral load in untreated HIV infection.

In the early years of the pandemic, HIV infection was a death sentence even in wealthy countries with good access to health care. Patients inevitably developed AIDS and died from opportunistic infections or malignancies on average approximately 10 years after seroconversion, ranging from <1 year to >20 years [12-15]. In 1987, the first antiretroviral drug, zidovudine, was introduced, raising hope of a cure. Shortly after, however, it was discovered that the HIV virus was capable of developing resistance to

zidovudine. Later, new antiretroviral drugs were released: didanosine in 1991, zalcitabine in 1992, stavudine in 1994 and lamivudine in 1995 [16], but most patients developed resistance successively to all new drugs given as monotherapy [17, 18].

Figure 1: Development of CD4 cell count, HIV RNA viral load and clinical symptoms after HIV infection (Source: Wikipedia)



From 1996, the introduction of a new class of antiretroviral drugs, protease inhibitors, and the concept of highly active antiretroviral therapy (HAART), a combination therapy of at least 3 different antiretroviral drugs, radically changed the prognosis for people living with HIV [19, 20]. HIV-infected individuals who receive HAART are now able to live normal lives with a life expectancy that might approach that of non-HIV-infected individuals, particularly when treatment is initiated before severe immunodeficiency has developed [21-23]. Moreover, HAART effectively prevents HIV transmission from

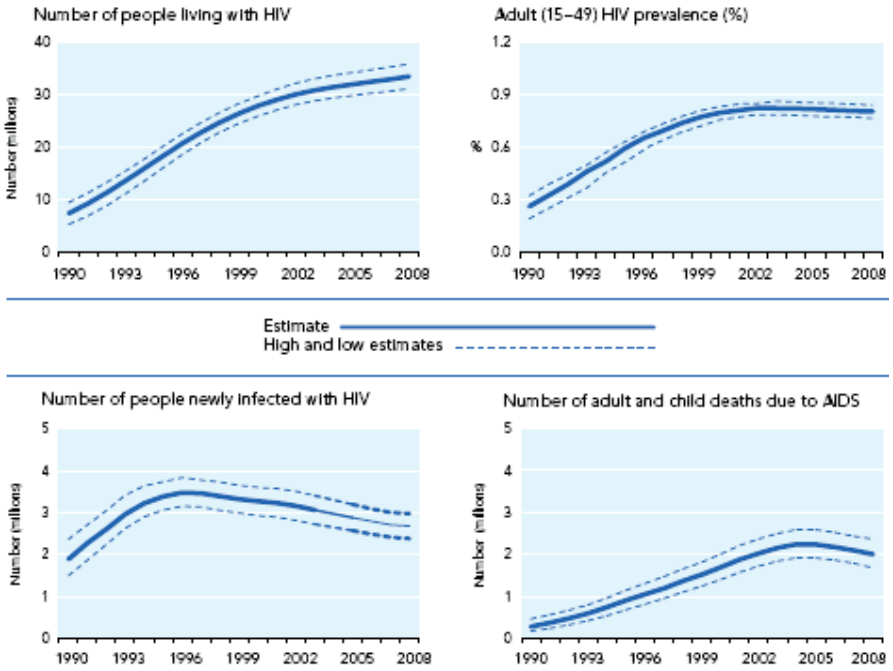
pregnant women to their infants, reducing the overall transmission risk through pregnancy, delivery and breastfeeding from 21-43% to approximately 2% [24-26].

1.2. Epidemiology

By the end of 2008, an estimated 33.4 million people were living with HIV/AIDS. Two million AIDS-related deaths occurred in 2008, and 2.7 million individuals were newly infected with HIV the same year. After a massive increase in the HIV prevalence and incidence in the 1980s and 1990s, the epidemic has stabilized over the past decade, and the number of new infections and AIDS-related deaths has decreased slightly (Figure 2) [9].

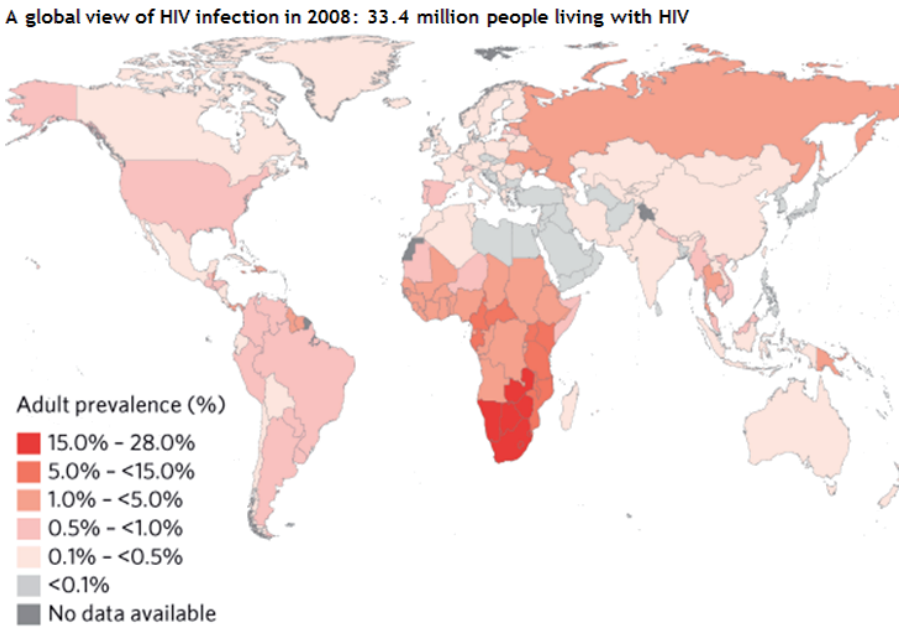
Figure 2: Global estimates of HIV prevalence, incidence and AIDS-related deaths

(Source: UNAIDS/WHO [9])



There are large geographical differences in the distribution of HIV/AIDS (Figure 3). In North America and Europe the epidemic has been concentrated to certain risk groups, mainly men who have sex with men, injecting drug users and immigrants. In sub-Saharan Africa the epidemic is generalized, and the majority is infected through heterosexual exposure. Africa is the continent hardest hit by the pandemic, with an adult prevalence of 14-26% in certain countries in Southern Africa [9].

Figure 3: Adults HIV prevalence in 2008, by country (Source: WHO)



Our study was carried out in Tanzania (figure 4), a low-income country in East Africa, which was ranked 151 of 182 in the latest Human Development Index [27]. The total population is estimated to be 39.7 million, of whom approximately 75% reside in rural areas [28]. Tanzania experiences a generalized HIV epidemic with an estimated adult HIV prevalence of 5.7%, which translates into 2.3 million people currently living with HIV/AIDS [9]. Life expectancy at birth is 46.5 years, which is estimated to be ten years lower than it would have been without HIV/AIDS [29].

Figure 4: Map of Tanzania

1.3. Mortality

HIV-related mortality declined dramatically in industrialized countries after the introduction of HAART in 1996 [19, 20]. At the beginning of 1998, death rates among HIV-infected individuals in Europe had fallen to less than a fifth of the pre-HAART level [19]. Still, mortality exceeds that of the general population, particularly when treatment is initiated late, i.e. with a history of AIDS and low CD4 cell count [21, 23]. Although AIDS remains the most common cause of death, other conditions such as cardiovascular

disease, liver disease, pulmonary disease and non-AIDS malignancy, have become increasingly important and account for nearly half of the deaths in people living with HIV in North America [30].

In resource-limited settings mortality data are scarcer. At the onset of the present study (in 2007), few results from routine antiretroviral treatment (ART) programs in Africa had been published. Some early studies showed overall good program performance, although with a high mortality, particularly the first months after initiating ART [31-37]. The higher early mortality in low-income compared to high-income countries seemed to be, at least partly, explained by later initiation of ART [38], and AIDS appeared to be the predominant cause of death [39].

Most studies on ART in Africa have been carried out in larger cities [32-34, 36, 37], often with support from an international non-governmental organization [31, 33, 35], and usually with short follow-up time [33, 35-37]. To date, there is a paucity of research from rural settings, which often face additional challenges, such as shortages of health workers, transport difficulties and other logistical constraints. In Tanzania, as in many other African countries, the majority of the population reside in rural areas [28]. In the present study, therefore, we aimed to assess long-term outcomes of ART in a rural Tanzanian hospital.

1.4. Antiretroviral treatment

Since the introduction of zidovudine in 1987, a number of new antiretroviral drugs have been released, and currently more than 25 individual drugs from six therapeutic classes are commercially available [16]. European guidelines recommend to start therapy with a backbone of two nucleoside reverse transcriptase inhibitors (NRTI), plus either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a ritonavir-boosted protease inhibitor [40].

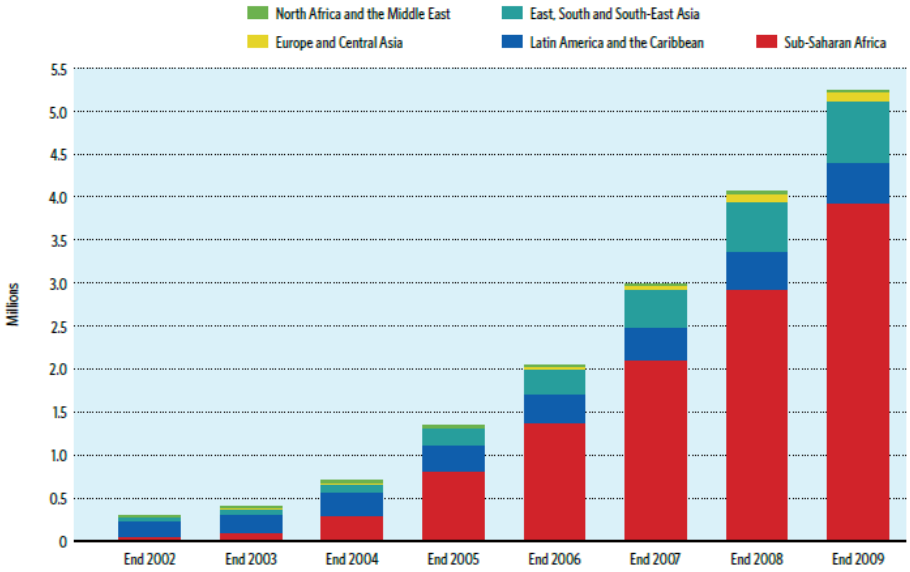
Until recently, the vast majority of HIV-infected individuals worldwide did not have access to ART due to poverty and lack of health care infrastructure. Over the past years, however, the World Health Organization (WHO), Joint United Nations Programme on HIV/AIDS (UNAIDS) and international non-governmental organizations have led a massive public health campaign aiming for universal access to ART. Simultaneously, generic competition has brought down prices of antiretroviral drugs from more than US$10,000 to less than US$70 per person per year, and the use of fixed-dose combination tablets has simplified treatment substantially [41].

In 2002, the WHO issued guidelines for scaling up ART in resource-limited settings, followed by revisions in 2003, 2006 and 2010 advocating earlier initiation of treatment [42-45]. The WHO guidelines recommend a first-line regimen based on two NRTIs in combination with an NNRTI. The most widely used ART regimen today is a fixed-dose combination (1 tablet morning and evening) of stavudine, lamivudine and nevirapine

[46]; however, the latest WHO guidelines recommend to move away from this regimen because of the long-term toxicity of stavudine [45].

By the end of 2009, more than 5 million people were receiving ART in low- and middle-income countries, which is a 13-fold expansion over the past 6 years (Figure 5) [46]. Inevitably, the long-term negative consequences of ART, including late drug toxicities, treatment failure and emergence of drug resistance, will become increasingly evident over the coming years [47-49]. Hence, as more and more people are receiving ART worldwide, huge challenges remain in terms of ensuring long-term efficacy of therapy.

Figure 5: Number of people receiving ART in low- and middle-income countries (Source: WHO/UNAIDS/UNICEF [46])



1.5. Resistance

Genomic heterogeneity is a key feature of HIV and new mutations occur frequently [50, 51]. Under pressure of a specific antiretroviral drug, mutations conferring resistance to this particular drug will have an evolutionary advantage and the mutant virus might overgrow the wild-type virus. In such a setting, the HIV viral load may increase and immunodeficiency develop despite ART [17, 18].

The key to long-term benefit of ART is sustained suppression of viral replication through provision of at least 3 different antiretroviral drugs given simultaneously [52-54]. In patients with suboptimal adherence or reduced serum drug concentrations for other reasons, selection of resistance can occur rapidly. The probability of drug resistance increases with duration of treatment, and in a study from UK nearly 30% harbored drug resistance after 6 years [55].

The NNRTIs and lamivudine/emtricitabine are particularly vulnerable to emergence of resistance since single-base mutations may be sufficient to induce high-level resistance [56]. In resource-limited settings, 99% initiate an NNRTI-based first-line regimen, and therefore resistance is likely to become an increasing problem in the coming decade [46]. Indeed, some have argued that scaling up ART in Africa could create widespread drug resistance [57, 58]. However, at the onset of the present study, there was a scarcity of studies presenting long-term resistance results from Africa, particularly from rural areas.

1.6. Monitoring of treatment

In high-income countries, monitoring of patients on ART with HIV-1 RNA viral load measurements and genotypic resistance testing is standard of care. Effective therapy should suppress the viral load to undetectable levels by 24 weeks and thereafter maintain full viral suppression. Patients with consistent viral load elevations during treatment should be tested for genotypic resistance, so that their ART regimen can be modified according to the resistance profile [40, 59].

Early detection of treatment failure and prompt switch to a fully active second-line regimen is key to prevent accumulation of drug-resistance mutations, which could jeopardize future drug options [60-62]. Furthermore, effective suppression of viral replication is crucial to avoid the negative consequences of HIV-infection [63], and a delayed switch after treatment failure is associated with increased mortality [64, 65].

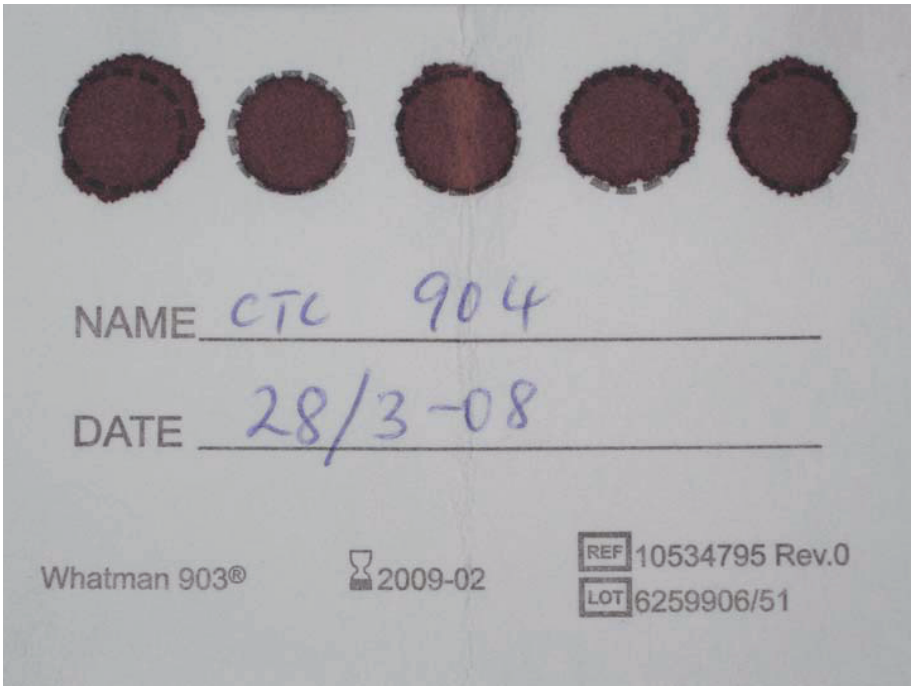
In resource-limited settings, however, viral load measurements and genotypic resistance testing are rarely available due to their high costs, complexity and stringent requirements for storage and transport of plasma. Hence, after a patient has initiated ART, it is often impossible to determine whether the treatment is effective or not. In settings without access to viral load monitoring, the WHO recommends to detect treatment failure by clinical (new or recurrent WHO stage 4 condition) or immunological (impaired CD4 cell response) criteria [44, 45]; however, recent studies have shown that these criteria have poor sensitivity and specificity in detecting true virological failure [66-70]. Using the WHO criteria, the majority of patients with treatment failure will not be detected until

they develop severe immunodeficiency or opportunistic disease, at which stage widespread resistance is likely [71, 72]. Furthermore, many patients will be misclassified as treatment failures despite adequate virological response, and risk premature switch to complex and expensive second-line therapy [73]. Thus, a vital question in the scale-up of ART remains unanswered: How can HIV viral load and drug resistance be monitored in settings with limited laboratory capacity?

1.7. Dried blood spots

Dried blood spots (DBS) can be a practical tool to overcome the challenges associated with storage and transport of blood specimens in settings with limited infrastructure. Plasma samples require a trained phlebotomist, electricity for centrifugation, a freezer for storage, and ultimately a functioning cold chain and speedy transport to the laboratory. DBS, on the other hand, are prepared by spotting whole-blood onto a filter paper, either from venous blood or directly from a finger prick, making this method particularly suitable in rural settings where laboratory personnel are scarce (Figure 6). Furthermore, DBS packed in zip-lock plastic bags with desiccant can be stored and shipped at ambient temperature, thus avoiding the requirement for a freezer and carriage on dry ice [74]. DBS specimens can be sent by truck or bus to the reference laboratory, or simply by the postal system where available. Biohazard risks associated with shipment are minimized since DBS cannot break and HIV on the filter paper matrix looses infectivity on drying [75].

Figure 6: Dried blood spots (DBS) on a Whatman 903 filter paper.



DBS have been used for more than 40 years to screen for metabolic disorders in neonates [76]. Recently, several African countries, including South Africa with the world's largest HIV-positive population, started to use DBS to screen for HIV in infants born of HIV-infected mothers. Detection of HIV-1 DNA in DBS is a highly sensitive and specific test of HIV-infection in infants [77]. Hence, the use of DBS is already familiar to health care providers and laboratory technicians in several low-income countries.

Prior to the onset of our study, a few studies had shown that DBS could be used to reliably measure HIV-1 RNA viral load [74, 78-81] and genotypic drug resistance [82].

However, these studies were carried out under standardized conditions in modern laboratories, and did not necessarily reflect real-life application in resource-limited settings. Hence, there was a need to validate the use of DBS for virological monitoring under field conditions in rural Africa, the setting where it would be of most use.

1.8. About Haydom Lutheran Hospital

Haydom Lutheran Hospital is a 400-bed hospital in Manyara region in northern Tanzania. It was founded by the Norwegian Lutheran Mission in 1953 and handed over to the Evangelical Lutheran Church of Tanzania 10 years later. The hospital is the main health care provider to a rural population of about 260,000 people, and available services include a fairly well equipped laboratory with microscopy, bacteriology and biochemistry, a modern radiology department with ultrasonography and computer tomography, as well as standard surgical and obstetrical services.

Adult HIV prevalence in the area is estimated to be 1.8% [83]. In 2002, the hospital launched a comprehensive HIV prevention and intervention program, funded by the Royal Norwegian Embassy and the US President's Emergency Plan for AIDS Relief (PEPFAR), with emphasis on voluntary testing and counselling through outreach services and antenatal clinics. An HIV Care and Treatment Centre was established adjacent to the hospital, and from October 2003 ART has been provided free of charge to eligible HIV-infected patients. Clinical officers, under supervision of a physician, have been responsible for medical follow-up of patients. On-site training was provided by HIV

specialists from collaborating institutions in Norway. A community home-based care network was established to follow-up adherence and trace missing patients.

Haydom Lutheran Hospital has always enjoyed good connections to Norway through financial support and visiting specialists. Furthermore, Haydom Lutheran Hospital has been involved in clinical research for several years through its collaboration with various Norwegian institutions, including University of Bergen, University of Oslo and Sørlandet Hospital. In 2007, the National Institute for Medical Research in Tanzania set up a research station at Haydom to coordinate the different research activities.

2. Aim and objectives

2.1. Main aim:

Assess the efficacy of modern antiretroviral treatment of HIV/AIDS at a rural hospital in Tanzania, and explore simplified laboratory methods for monitoring of treatment.

2.2. Specific objectives:

- Assess mortality and other program losses among patients who initiate ART (Paper I).
- Study virological efficacy and emergence of drug resistance in patients who receive ART (Paper II).
- Identify predictors of mortality and drug resistance in patients who initiate ART (Paper I and II)
- Validate the use of dried blood spots under field conditions for monitoring of HIV-1 RNA viral load (Paper III).
- Validate the use of dried blood spots under field conditions for genotypic resistance testing (Paper IV).

3. Material and methods

3.1. Patients, treatment and follow-up

Most of the patients enrolled in the HIV program in Haydom were diagnosed either through HIV testing and counselling in the villages or they were hospitalized patients tested on clinical suspicion. Therefore, many of the patients had advanced immunodeficiency at the time of enrolment into HIV care. In our study we decided to include all patients regardless of presumed prognosis. This strategy was adopted to get a representative picture of a routine ART program in rural Tanzania.

ART was initiated in accordance with guidelines from the WHO and the National AIDS Control Program [42-44, 84]: WHO stage 4 irrespective of CD4 cell count, WHO stage 3 with CD4 ≤350 cells/μL, or CD4 ≤200 cells/μL with any WHO stage. However, reliable CD4 cell counts were not available until September 2006; thus, most patients started ART based on clinical criteria only (WHO stage 3 or 4). In addition, triple-drug combination ART, and not single-dose nevirapine which has been used widely in other programs, was offered to HIV-infected pregnant and lactating women, from pregnancy week 20 till cessation of breast-feeding, irrespective of WHO stage and CD4 cell count, to prevent mother-to-child transmission.

First-line treatment comprised stavudine or zidovudine, combined with lamivudine, and either nevirapine or efavirenz. Regimen choice was subject to availability, with use of a generic fixed-dose combination of stavudine, lamivudine and nevirapine whenever possible. Second-line treatment was available from December 2006 and comprised

lopinavir/ritonavir, didanosine and abacavir. Criteria for switching to second-line ART was virological failure as recommended in the 2006 revision of the WHO guidelines (i.e. viral load >10,000 copies/mL) [44]; however, viral load was not measured routinely, and only selected patients with high clinical suspicion of failure were tested. Patients with CD4 ≤200 cells/μL or WHO stage 3 or 4 disease were given co-trimoxazole prophylaxis 960 mg thrice weekly or 480 mg daily. After the initial 2 weeks of daily drug administration, antiretroviral drugs were dispensed on a monthly basis. Patients were seen by a clinical officer every 3 months, and CD4 cell counts were performed every 3-6 months.

3.2. Data collection

Data for the study was collected from the patient files at the HIV Care and Treatment Centre. A standardized form was used for the baseline evaluation, which included socio-demographic information, medical history, physical examination and laboratory investigations (Appendix). Clinical staging was performed using the most updated revision of the WHO clinical staging system [42-44].

Deaths were registered from hospital records or reported through home visitors. The home visitors recorded date of death and likely death cause (or symptoms preceding death) by interviewing relatives of the deceased. There is no central death register in Tanzania, making the assessment difficult in patients who moved out of the hospital's catchment area, but the clinic staff attempted to trace all patients who failed to attend the

clinic. Patients who missed appointments for more than 3 months, and could not be traced by the home visitor, were regarded lost to follow-up.

3.3. Laboratory methods

3.3.1. Routine tests performed at Haydom Lutheran Hospital

Standard laboratory investigations at baseline included: Full blood cell count, CD4 cell count, erythrocyte sedimentation rate, liver function tests, creatinine, blood sugar, hepatitis B surface antigen and syphilis serology.

HIV infection was established using two different rapid antibody tests. Standard hematology was measured using Sysmex KX-21 Hematology Analyzer (Sysmex Corp., Kobe, Japan). CD4 cell counts were initially measured by manual techniques (Dynabeads CD4; Dynal Biotech ASA, Oslo, Norway); however, results were observed to be unreliable, and from September 2006 an automated flow cytometer (FACSCount; Becton Dickinson, San Jose, CA, USA) was employed. For the purpose of this study only CD4 cell counts from the FACSCount were considered.

Body mass index (BMI, weight in kilograms divided by height in meters squared) was used to assess nutritional status. Body weight was measured at each clinic visit using the same manual scale, and height was measured at baseline using a stadiometer mounted on the scale.

3.3.2. HIV-1 RNA viral load

HIV-1 RNA viral load was not a routine test in the program, but was performed as part of the current study in patients who had completed at least 6 months of first-line ART. Blood was collected on plasma preparation tubes (PPT; Becton Dickinson, Franklin Lakes, NJ, USA) by venous puncture and centrifuged within 3 hours. Plasma was immediately transferred to sterile plastic tubes and stored at -20°C. Manufacturer's instructions were followed with regard to sample collection, storage and transport. Viral load was measured at Muhimbili National Hospital, Dar es Salaam, Tanzania, using the Cobas TaqMan 48 Analyzer (Roche Diagnostics, Branchburg, NJ, USA) with a lower detection limit at 40 copies/mL. However, due to equipment breakdown, a subset of samples were analysed with the Cobas Amplicor HIV-1 Monitor v1.5 (Roche Diagnostics) with a detection limit at 400 copies/mL.

As a quality control, 7 random duplicate samples were sent without prior freezing to Oslo University Hospital and tested within 48 hours of sample collection, using the same viral load assay (Cobas TaqMan 48 Analyzer; Roche Diagnostics). Results from Oslo University Hospital showed good agreement with samples tested at Muhimbili National Hospital after 5 weeks storage in Haydom at -20°C (Table 1); the mean difference was only 0.06 $\log_{10}$ copies/mL (standard deviation [SD] 0.22).

Table 1: Comparison of viral load results (log_{10} copies/mL) from Oslo University Hospital (OUS) and Muhimbili National Hospital (MNH) in 7 duplicate plasma specimens

	1	2	3	4	5	6	7	**Mean**
MNH	5.20	5.81	3.42	5.32	3.31	5.96	6.43	**4.95**
OUS	5.23	5.69	3.71	5.17	3.73	6.00	6.34	**5.01**
Difference	-0.03	0.12	-0.29	0.15	-0.42	-0.04	0.09	**-0.06**

3.3.3. Genotypic resistance testing

Resistance testing was not available in Tanzania at the time of our study. For the purpose of this study we were granted permission to ship samples to Oslo University Hospital for genotyping. Plasma was genotyped using the routine method at Oslo University Hospital, which comprises the ViroSeq HIV-1 Genotyping System v2.0 (Abbott Molecular, Des Plains, IL, USA) with inclusion of an in-house reverse transcriptase (RT)-nested polymerase chain reaction (PCR) step, as described in detail in paper II and IV.

3.3.4. Dried blood spots

DBS were prepared in parallel with plasma from the same blood samples. Whole-blood from a PPT tube was spotted onto two Whatman 903 filter paper cards (Whatman plc, Maidstone, UK) to completely fill the circles. The cards were then left to air-dry overnight, and stored in zip-lock plastic dispensing bags (purchased locally) with a silica

desiccant (Elcon-Broker AS, Holmestrand, Norway). The two DBS cards were stored and processed differently:

1) Stored locally at -20°C, but exposed to ambient temperature for 20 days during transport: these samples were used for the viral load study (Paper III).
2) Stored and shipped at ambient temperature: these samples were used for the resistance study (Paper IV).

In order to extract and isolate nucleic acids from the filter papers, we used the NucliSens silica-based Boom extraction method (BioMérieux, Inc., Durham, NC, USA) with some modifications for DBS processing [85, 86]. The procedure is described in detail in paper III and IV.

Viral load quantification from DBS was performed using the NucliSens EasyQ HIV-1 v1.2 (BioMérieux), which comprises nucleic acid sequence-based amplification (NASBA) and real-time detection using molecular beacons targeting the *gag* gene. The NASBA method is an isothermal amplification (41ºC), which specifically amplifies single-stranded RNA, by the use of T7 RNA polymerase. It has a linear dynamic range from 50 to 3,000,000 IU/mL when 1 mL of plasma is used [87].

Genotyping from DBS, after manual extraction and isolation of nucleic acids, was carried out using the same method as for plasma, as described above.

3.4. Study design

HIV-infected individuals who started ART were included in a prospective cohort study. Specific inclusion and exclusion criteria are listed in each paper.

Paper I and II were epidemiological studies:

- Paper I assessed mortality in an open cohort of adults (≥15 years) who successively started ART. A longitudinal cohort study is well suited to describe mortality and investigate possible associations between various factors and death [88]. Women who started ART exclusively to prevent mother-to-child transmission, and not for their own health, were excluded from this paper, since they were essentially a different population than those who started ART due to immunodeficiency.
- Paper II assessed virological efficacy of ART in a cross-sectional survey of adults (≥15 years) who had completed at least 6 months of first-line ART. We aimed to include all patients on ART at the time of the survey, but excluded those who for various reasons had stopped ART. A cross-sectional survey is suitable to determine the prevalence of various conditions, in this case virological failure and drug resistance [89].

Paper III and IV were experimental laboratory studies:

- Paper III compared HIV-1 RNA viral loads in DBS with results from a plasma-based method, using samples obtained from patients on ART.

- Paper IV compared HIV-1 genotypes derived from DBS with those of plasma, using samples from patients with treatment failure (viral load >1000 copies/mL).

3.5. Statistical analysis

We used standard techniques of survival analysis to estimate mortality and identify predictors of mortality (Paper I). Kaplan-Meier models were used to estimate mortality after ART initiation, and log rank tests to compare survival curves. Cox proportional hazards models were used to identify independent predictors of mortality and calculate hazard ratios [88].

Logistic regression was used to study associations between baseline characteristics and emergence of drug resistance (Paper II). Since this part of our study was based on a cross-sectional survey, we could not ascertain when resistance mutations had emerged, and therefore techniques of survival analysis would not be appropriate [88].

We used linear regression analysis on $\log_{10}$-transformed data to compare viral load results in DBS and plasma, and employed the analysis described by Bland and Altman to describe agreement between the two methods (Paper III) [90]. Thereafter, to assess the ability of DBS to identify patients with major virological failure (≥5000 copies/mL), we used Receiver Operating Characteristics (ROC) curves to identify the optimal threshold in DBS and the corresponding sensitivity and specificity. The area under the ROC curve ranges from 0.5 in a completely random test to 1.0 when there is perfect agreement, and this method provides a graphical representation of the tradeoff between sensitivity and

specificity. ROC curves are commonly used for the purpose of assessing a new diagnostic method against the standard method [91].

Finally, we used simple descriptive statistics to compare resistance mutations in DBS and plasma with regard to amplification success, detection of mutations, and nucleotide similarity (Paper IV). Logistic regression was used to study factors associated with amplification failure in DBS.

Data were analyzed using SPSS (version 14.0-16.0) for Windows (SPSS Inc., Chicago, IL, USA), except 95% confidence intervals for proportions which were calculated with NCSS version 2007 (NCSS, Kaysville, UT, USA). All tests were two-sided and level of significance was set at $P < 0.05$.

3.6. Ethical considerations

Ethical approval, including permission to take blood samples to Norway, was obtained from the Medical Research Coordinating Committee of the National Institute for Medical Research (NIMR) in Tanzania and Regional Committee for Medical Research Ethics (REK-Øst) in Norway. Permission to carry out the research was granted by the hospital management at Haydom Lutheran Hospital and Personvernombudet at Oslo University Hospital. Patients gave written consent to participate in the study.

We performed viral load and resistance testing on a number of patients, and every effort was taken to make sure the test result was communicated back to the clinician in charge

and proper action taken. This would sometimes involve purchasing second-line antiretroviral drugs or other medicines not available locally for selected patients. We consider this an obligation of medical research in low-income countries.

It felt like a heavy responsibility to perform research involving patients who were often critically ill. During the data collection period in Haydom, I felt obliged to participate in the clinical work at the hospital in order to contribute with my medical skills in a rural hospital with a desperate need for healthcare professionals. The clinical work at the hospital was truly one of the most rewarding experiences as a PhD fellow.

4. Synopsis of results

4.1. Paper I: Predictors of mortality in HIV-infected patients starting antiretroviral therapy in a rural hospital in Tanzania

This paper aimed to assess mortality and to identify predictors of mortality among individuals who started ART. Of 320 patients included, 223 (69.7%) were women and median age was 35 years (interquartile range [IQR] 30-43). At ART initiation, 210 patients (65.6%) had clinical AIDS (WHO stage 4). After a median follow-up period of 10.9 months (IQR 2.9-19.5) we found that 95 of 320 (29.7%) patients had died, of whom 59 died within the first 3 months after starting ART. Kaplan-Meier estimated survival was 0.808, 0.710, 0.648 and 0.593 after 3, 12, 24 and 36 months, respectively. In a Cox proportional hazards model the following baseline variables were independent predictors of mortality:

- severe anemia (hemoglobin <8 g/dL; adjusted hazard ratio [AHR] 9.20; 95% confidence interval [CI] 2.05-41.3)
- moderate anemia (hemoglobin 8-9.9 g/dL; AHR 7.50; 95% CI 1.77-31.9)
- thrombocytopenia (platelet count <150 x 10^9/L; AHR 2.30; 95% CI 1.33-3.99)
- severe malnutrition (BMI <16 kg/m^2; AHR 2.12; 95% CI 1.06-4.24).

A simple prognostic model based on hemoglobin level appeared to be a useful tool for initial risk assessment in this setting: Estimated one year mortality was 3.7% in patients without anemia, 20.0% in mild anemia, 37.6% in moderate anemia and 55.2% in severe anemia (P <0.001). A similar trend was observed with decreasing BMI: Estimated one

year mortality was 13.7% in patients with normal nutritional status, 21.0% in mild to moderate malnutrition, and 46.8% in severe malnutrition (P <0.001).

In conclusion, this study found a high early mortality among patients who started ART, and anemia, thrombocytopenia and low BMI were strong predictors of mortality.

4.2. Paper II: Virological efficacy and emergence of drug resistance in adults on antiretroviral treatment in rural Tanzania

In this paper we assessed virological efficacy and emergence of genotypic resistance among 212 patients who had received ART for at least 6 months (median follow-up time 22.3 months; IQR 14.0-29.9). Viral suppression (viral load <400 copies/mL) was observed in 187 patients (88.2%). The proportion of patients (95% CI) with suppressed viraemia after 1, 2, 3 and 4 years was 94.8% (87.2-98.6), 88.0% (79.0-94.1), 75.0% (57.8-87.9) and 87.5% (61.7-98.4), respectively.

Genotyping was successful in 22 of 23 samples with viral load >1000 copies/mL, of which 18 samples (82%) harbored at least one clinically relevant resistance mutation in the RT gene. The most frequent mutations were: M184I/V (n=14), conferring resistance to lamivudine/emtricitabine, and K103N (n=6), Y181C (n=6) and G190A (n=6), conferring resistance to NNRTIs. Fourteen patients had dual-class resistance, i.e. resistance to both NRTIs and NNRTIs. Thymidine analogue mutations (TAMs), associated with broad cross-resistance to NRTIs, were found in 5 patients. In total, 18 of

212 patients (8.5%) harbored drug resistance by use of a standard genotyping assay. The prevalence (95% CI) of any clinically significant drug resistance after 1, 2, 3 and 4 years was 3.9% (0.8-11.0), 8.4% (3.5-16.6), 16.7% (6.4-32.8) and 12.5% (1.6-38.3), respectively. Only duration of ART was significantly associated with emergence of drug resistance (≥3 years vs. 1 year on ART; odds ratio [OR] 4.49; 95% CI 1.13-17.8; *P*=0.033).

We concluded that virological suppression rates were good up to 4 years after starting ART; however, of concern, drug resistance increased with time.

4.3. Paper III: Dried Blood Spots perform well in viral load monitoring of patients who receive antiretroviral treatment in rural Tanzania

This paper aimed to compare HIV-1 RNA viral loads from DBS with results of a plasma-based method. We obtained 98 plasma-DBS pairs from patients on ART, with plasma viral loads ranging from undetectable to >1,000,000 copies/mL. In a linear regression model there was a strong correlation between HIV-1 RNA levels in plasma and DBS, with a slope of 0.76, an intercept of 0.69, and an R^2 value of 0.75. HIV-1 RNA levels were, on average, slightly higher in plasma compared to DBS, but mean difference was only 0.04 $\log_{10}$ (SD 0.57). Eight samples yielded more than 1 $\log_{10}$ difference between plasma and DBS values.

Sensitivity using DBS was reduced in samples with low-level viraemia. In samples with plasma viral loads ranging from 40 to 1000 copies/mL, only 2 of 28 (7%) had detectable HIV-1 RNA in DBS. On the contrary, all 27 samples with plasma viral loads ≥3000 copies/mL had detectable HIV-1 RNA in DBS.

The ability of DBS to detect major virological failure (≥5000 copies/mL) was assessed using ROC curves. Area under the ROC curve was 0.97, indicating that DBS had strong diagnostic properties. The optimal DBS threshold appeared to be at 5000 IU/mL, giving a sensitivity, specificity, positive predictive value and negative predictive value of 0.91, 0.97, 0.79 and 0.99, respectively (assuming 10% major virological failure). Ninety-four of 98 plasma/DBS pairs (96%) showed concordant results.

We concluded that DBS performed well in viral load quantification of patients on ART in rural Tanzania, even though sensitivity was reduced in patients with low-burden viraemia.

4.4. Paper IV: HIV type-1 drug resistance testing on dried blood spots is feasible and reliable in patients who fail antiretroviral therapy in rural Tanzania

This paper aimed to compare genotypic resistance results from DBS with those of plasma. We included 36 ART-experienced individuals with treatment failure (viral load >1000 copies/mL) and an available plasma genotype. HIV-1 *pol* was successfully

amplified in 34 of 36 (94%) corresponding DBS specimens. The two DBS specimens which could not be amplified both had low-level viraemia: 1432 copies/mL and 2621 copies/mL.

Among 34 plasma/DBS pairs, the mean nucleotide similarity was 98.1% (SD 0.87). In the protease region, 142 mutations were detected in plasma, of which 132 (93%) were also found in DBS. Five protease mutations were detected in DBS only, all of which were amino acid mixtures with the wild-type strain. All the plasma-derived protease mutations were minor mutations or polymorphisms, and none of the patients harbored resistance to protease inhibitors. In the RT region, however, 57 clinically significant mutations were found in plasma, of which 51 (89%) were also detected in DBS. Four of the 6 RT mutations that were missed in DBS were from patients with early treatment failure (viral load ≤3000 copies/mL). Two RT mutations were detected in DBS only, but both were amino acid mixtures in the 184 position with the same clinical interpretation: M184IMV in DBS and M184V in plasma. Thirty of 34 (88%) patients had identical resistance profiles to antiretroviral drugs in plasma and DBS, according to the Stanford interpretation.

In conclusion, genotyping from DBS was successful in the vast majority of samples, and there was a high concordance between mutations found in plasma and DBS.

5. Discussion

Although a number of ART studies have been conducted in sub-Saharan Africa over the past few years, there is still a paucity of research from rural settings. The scale-up of ART in resource-limited settings began in larger urban centres [32-34, 36, 92], and still services to rural populations lag behind [93]. Rural ART programs face a number of additional challenges, such as shortages of healthcare personnel, transport difficulties and other logistical constraints, and experiences from well-run clinics in Cape Town or Lusaka are not necessarily generalizable to rural Tanzania. The present study is one of few to report results from a routine ART clinic in rural Africa, and we believe our findings better reflect the realities in such settings.

In the first part of this study we assessed overall program performance, including mortality, viral suppression and emergence of drug resistance. We found a very high mortality in this cohort, particularly the first months after initiating ART, which at least partly could be explained by advanced immunodeficiency at baseline. On the other hand, among patients who survived the first 6 months and remained in care, long-term virological suppression rates were good and the overall prevalence of drug resistance was relatively low. Our results suggest that long-term benefits of ART can be sustained in rural Tanzania, but treatment should be initiated before patients have developed AIDS. Furthermore, we sought to identify clinically useful predictors of mortality, so that patients at particularly high risk could be identified at an early stage. Anemia, malnutrition and thrombocytopenia were strong and independent predictors of mortality in our cohort. A prognostic model based on hemoglobin level appeared to be useful for

initial risk assessment, and can be of particular use in settings without access to CD4 cell counts.

In the second part of the study we aimed to find a solution to one of the most pressing problems we encountered in our setting: the lack of field-friendly assays for viral load quantification and drug resistance testing. The use of DBS could simplify blood collection and shipment, and in our study there was a good agreement between viral load levels in DBS and plasma, although DBS had reduced sensitivity in samples with low-level viraemia. Furthermore, in patients with treatment failure, resistance genotyping was successful in the vast majority of DBS specimens, and there was high concordance between mutations found in DBS and plasma. Our study suggests that DBS can be a feasible and reliable tool to monitor viral load and drug resistance in patients on ART in resource-limited settings.

In the following I elaborate on our main findings, their strengths and weaknesses, and their consequences for clinical practice.

5.1. Sample and representativeness

An important question is: How representative are our findings? Haydom Lutheran Hospital is an old mission hospital which has benefited from support from Norway for more than 5 decades, and as such might not be representative of all rural hospitals in Tanzania. Certain advanced equipment available in Haydom, such as computer tomography for suspected cerebral toxoplasmosis, is definitely unavailable in most rural

hospitals in the country. Furthermore, reliable CD4 cell counts were available already in 2006, at a time when this was rare in other rural hospitals.

On the other hand, the patients included in our study, in an area where the majority rely on subsistence farming and pastoralism, are typical of rural Africa. Furthermore, Haydom Lutheran Hospital is faced with the same logistical challenges as other rural hospitals in the country due to its remote location and high patient load. Although the hospital is church-owned, the HIV program in Haydom is fully integrated in the National AIDS Control Program at the Ministry of Health and Social Welfare. Moreover, approximately half of the hospital beds and rural health services in Tanzania are operated by churches, and thus, being a church-owned hospital is not atypical.

Obviously, hospitals in Tanzania and Africa differ hugely, and our findings are not necessarily applicable to all locations. However, we believe that our data better represent the situation in rural Africa than studies carried out in larger urban centres.

5.2. Mortality

We found a very high mortality in this cohort, with an estimated 1 year mortality of 29%. The majority of deaths occurred within 3 months of starting ART. Other studies from the region have also reported high early mortality; indeed, in a review article from 2008 Lawn and colleagues reported that between 8 and 26% of patients in sub-Saharan Africa die in their first year of ART, of whom the majority die in the first few months after ART initiation [94].

The higher mortality in our study compared to many other studies could have several explanations. First, being a hospital-based study, there was probably a bias towards more severe disease at baseline in our study compared to out-patient programs. Furthermore, this was the first ART program in the area, and in the early days of the program we might have included a "pool" of patients with severe AIDS, who thereby contributed to the poorer overall survival. Finally, we initiated ART in all eligible patients regardless of presumed prognosis, as opposed to certain other studies that excluded patients with active opportunistic infections, laboratory abnormalities or presumed poor adherence [95]. Moreover, some studies had a lengthy (4 weeks to 4 months) patient preparation process between enrolment and ART initiation, and consequently many of the most severely ill patients died pre-ART and did not contribute to on-treatment mortality [31, 33, 39]. Such a strategy gives a skewed impression of program performance.

The high early mortality rates in sub-Saharan Africa probably reflect advanced immunodeficiency at baseline. In our study, more than half of the patients had clinical AIDS at enrolment. In the ART-LINC collaboration (Antiretroviral Treatment in Lower Income Countries) the median CD4 cell count at ART initiation in sub-Saharan Africa was 111 cells/μL and 62% had advanced disease (WHO stage 3 or 4) [96]. By contrast, patients from Europe and North America in the ART-CC study (Antiretroviral Treatment Cohort Collaboration) initiated ART with a median CD4 cell count of 224 cells/μL and only 23% had advanced disease (CDC stage C) [97]. Our results and results from the ART-LINC study underscore the need to identify HIV-infected individuals and start

treatment earlier in the course of their illness. Likewise, as the majority of HIV-infected individuals are unaware of their HIV status [93], it is necessary to increase the availability and uptake of HIV testing and counselling services. Once patients have enrolled into HIV care, health system delays before ART initiation should be minimized. Indeed, in a recent study from sub-Saharan Africa, Brinkhof and colleagues showed that excess mortality is moderate and reaches that of the general population in the second year of ART, among HIV-infected individuals who initiate ART before severe immunodeficiency has developed, i.e. with less advanced disease and a CD4 cell count above 200 cells/μL [22].

We found that baseline anemia, malnutrition and thrombocytopenia were strong and independent predictors of mortality. Previous studies have also reported that anemia [32, 36, 98-101] and malnutrition [31, 35, 36, 102-105] predicts mortality, both in high-income and low-income countries. A new and important finding of our study, however, was that a prognostic model based on hemoglobin level appeared to be a particularly useful tool for initial risk assessment. By categorizing the patients according to baseline hemoglobin, we were able to distinguish between patients at low, low intermediate, high intermediate and high risk of death. Previously, Mocroft and colleagues have reported similar findings in European HIV patients, even after adjusting for CD4 cell count and viral load level [100]. Recently, studies from Côte d'Ivoire, South Africa and Malawi have confirmed the usefulness of hemoglobin level in prognostic models in African HIV patients [106, 107]. In our study, patients with a hemoglobin level below 8 g/dL had approximately 40% risk of death within 3 months of ART initiation. It is likely that these

early deaths were caused mainly by conditions preexisting at enrolment and/or by immune reconstitution syndrome [94]. Hence, in settings with limited resources, baseline hemoglobin level could be used to identify patients at high risk who would need a more targeted search for opportunistic infections and a closer follow-up. On the opposite, time and resources could be released by a less rigorous follow-up of patients at low risk. Hemoglobin is a cheap and simple laboratory test, and could be a particularly useful tool in settings without access to CD4 cell counts.

There were some weaknesses of our study. First, attrition rates were high: 10.9% were transferred out and 9.7% were lost to follow-up. It is likely that many of the patients classified as lost to follow-up in reality died, and thus the overall number of deaths is probably underestimated. The true mortality rates in this cohort could therefore be even higher than reported in our study.

The high attrition rates could also have affected the Cox proportional hazards models used to identify predictors of mortality. Patients lost to follow-up were censored at their last clinic visit, but if mortality was higher in individuals whose follow-up time was censored than it was in otherwise identical individuals whose follow-up time was not censored (termed informative censoring), then our results could be biased [88]. In a recent study on determinants of survival among patients on ART in Uganda, the authors found that not accounting for losses to follow-up led to identification of spurious associations and missed actual relationships [108]. Although losses to follow up in the

Ugandan study (22.9%) were much higher than in ours (9.7%), we can not exclude that our results were affected by a similar bias.

Furthermore, we lacked CD4 cell counts and HIV viral loads, which are important predictors of mortality in patients on ART [20]. The lack of these variables in the final Cox model might have impaired the generalizability of our results [109]. Nevertheless, our study suggests that alternative markers could be used for initial risk assessment in the absence of CD4 cell counts and viral loads.

Finally, we did not have reliable information on death causes, which would have been a nice addition to this study. Available studies from sub-Saharan Africa suggest that tuberculosis, cryptococcal meningitis, sepsis, Kaposi's sarcoma and wasting syndrome are the leading causes of death after ART initiation [94]; however, data are limited. Moreover, we did not systematically register toxicities of antiretroviral drugs. The long-term use of stavudine is a concern in resource-limited settings [37, 49, 110], and a systematic screening for lipodystrophy and neuropathy would be of interest in a cohort like ours. Future studies should aim to include this information.

5.3. Virological failure and resistance

Among patients alive and in care, virological suppression rates were good up to 4 years after initiating ART. In fact, our results with viral suppression in 94.8%, 88.0%, 75.0% and 87.5% of patients after 1, 2, 3 and 4 years, respectively, were comparable to results from the ART-CC study, in which 78% of patients in North America and Europe were

virologically suppressed after 3 years on ART [111]. Other studies from sub-Saharan Africa have usually had shorter observation time, but a few reports on long-term virological efficacy support our findings: a study from Senegal found viral suppression in approximately 70% after 2-3 years on ART [112], whereas a study from Botswana reported viral suppression in approximately 90% of patients throughout 5 years of observation [113]. Furthermore, a recent study from Khayelitsha township in South Africa found viral suppression in 83.8% after 5 years on ART [114]. It should be noted, however, that results from study settings might be better than results from routine ART programs, and therefore, more studies like ours are needed from routine clinical settings in rural Africa.

A key question in the global scale-up of ART is: Does drug resistance develop more rapidly in resource-limited settings than in industrialized countries? In our study we found that emergence of resistance increased with time and reached about 15% after 3-4 years on ART. A few other studies from sub-Saharan Africa have reported long-term resistance results. An early study from Senegal reported 12.5% resistance after a median of 30 months on ART [112], whereas a study from Côte d'Ivoire found 22% resistance after a median of 37 months [115]. For comparison, a study from Canada found drug resistance in 20% of patients after 30 months of treatment with stavudine/lamivudine/nevirapine, the regimen most widely used in resource-limited settings [116]. Furthermore, in a routine clinical setting in the UK, 19% harbored drug resistance after 4 years on ART [55]. An important conclusion of our study, therefore, is

that resistance seems to occur at a similar rate in rural Tanzania as in high-income countries.

Of concern, we found TAMs, associated with broad cross-resistance to NRTIs, in 23% of patients with treatment failure. Given the lack of routine viral loads in our program, it is likely that many of the patients had been treated with a failing ART regimen for months - or even years – before the survey, and prolonged exposure to a failing regimen is a known risk factor for accumulation of resistance mutations [60-62]. A much cited study by Phillips and colleagues used a computer simulation model to show that viral load monitoring of patients on ART in resource-limited settings only gives a slight survival benefit compared to clinical monitoring [117]. Some have used this article to argue that viral load monitoring is unnecessary; however, the same study also found that accumulation and transmission of resistance mutations occur more frequently in patients without viral load monitoring. More recently, a large meta-analysis found that emergence of TAMs, as well as other resistance mutations, was significantly more common in patients without access to regular viral load monitoring [118]. Hence, there is a need for a simple, affordable viral load assay adapted for use in tropical environments, so that treatment failure can be detected before multiple mutations occur.

In our study only duration of ART was significantly associated with drug resistance. Patients who had received ART for ≥3 years had more than 4-fold increased risk of harboring drug-resistance mutations compared to patients on ART for 1 year. This is in line with studies from Europe and North America [55, 119]. Moreover, we observed a

strong, but only borderline significant, association between baseline anemia and lymphopenia and emergence of resistance. This association should be confirmed in larger studies, but it might reflect the previously reported increased risk of drug resistance in patients who initiate ART with advanced immunodeficiency (high viral load and low CD4 cell count) [119]. Unfortunately, we lacked data on adherence, which is considered the major determinant of drug resistance in patients on ART [119].

Although overall results from our study were encouraging, with good virological suppression rates and moderate emergence of resistance, the economical and logistical consequences of treatment failure and drug resistance in resource-limited settings are considerable. With more than 5 million individuals currently receiving ART in low- and middle-income countries, 15% with drug resistance after 3-4 years of treatment, as observed in our study, translates into 750,000 patients in need of second-line ART within a few years. At present the cheapest WHO recommended second-line regimen (atazanavir/ritonavir + tenofovir/lamivudine) costs at least 7 times more than the cheapest first-line regimen [41]. Furthermore, the pill burden increases to at least 4 tablets daily, which might come in addition to treatment for tuberculosis or other opportunistic infections, and a high pill burden is associated with reduced adherence [120]. Our results suggest that funding for ART programs will have to increase substantially over the coming years in order to meet the increasing demand for second-line regimens. Moreover, newer antiretroviral drugs should be made available at affordable prices for patients who need third-line regimens, and new fixed-dose combination tablets should be developed in order to reduce pill burden and promote adherence.

There were certain limitations of the present study. Most importantly, as this was a cross-sectional virological survey of patients alive and in care, we left out patients who were transferred out, lost to follow-up, died or who discontinued treatment. Among patients who discontinued treatment (4.9%) or were lost to follow-up (8.4%), there was probably a substantial proportion who harbored drug resistance. Patients who were transferred out when ART became available through the public sector, however, did probably not differ significantly from those who remained in care. Among patients who died, the majority died within a few months of starting ART, most likely due to advanced disease at baseline rather than drug resistance. Our analysis, therefore, must be considered an on-treatment analysis, and results from an intention-to-treat analysis, which is often used in clinical trials, would have been poorer. However, our study is one of the first to provide information about long-term virological efficacy of ART in a rural African setting, and might provide a useful forecast of drug resistance and demand for second-line antiretroviral drugs in rural Africa in the coming years.

Another limitation of our study is that we based our viral load results on a single blood test. Viral load can increase during intercurrent illnesses or due to random biological and statistical variations ("blips"). Many studies define treatment failure as two consecutive viral loads above a set level, which is more robust to random variations. The use of a single viral load measurement in our study might therefore have led to a slight overestimation of virological failure; however, "blips" above 400 copies/mL, the failure threshold in our study, are rare [121].

With regard to the prevalence of drug resistance, our results might have been underestimated by selection and misclassification bias. Firstly, three patients who were already on second-line ART at the time of the survey were excluded since genotypic resistance results prior to the regimen switch were unavailable. Two of these had confirmed virological failure prior to the switch, whereas the third patient had dubious clinical failure without virological confirmation. Secondly, two patients with low-level viraemia (400-1000 copies/mL) and one patient whose genotyping failed were assumed not to harbour resistance. In the worst case, if all these six patients harbored drug-resistance mutations, the overall proportion with drug resistance would be 24 of 215 patients (11.2%), and the prevalence of drug resistance after 1, 2, 3 and 4 years would increase to 3.9%, 10.7%, 24.3% and 17.6%, respectively.

Since we lacked longitudinal virological data, we could not exclude that resistance was present pre-ART. However, Haydom Lutheran Hospital was the first centre to offer ART in the area, and it is unlikely that any of the patients had been exposed to previous ART. Transmitted resistance is still very rare in Tanzania, even in Dar es Salaam, where access to ART is better than in the rest of the country [122].

Finally, it is important to ask whether our virological results were reliable or not. Could suboptimal handling of blood samples have caused any systematic error in our results? HIV-1 RNA is vulnerable to degradation, and therefore, we paid strict attention to the manufacturer's instructions for collection, storage and transport of specimens. The

temperature in the freezer at the collection site was checked daily, and although power supply interruptions were frequent, the hospital's generator maintained the temperature constant. As described in chapter 3.3.2, we sent 7 duplicate samples without prior freezing to Oslo University Hospital, and found good agreement with samples stored for 5 weeks at -20°C in Haydom and tested at Muhimbili National Hospital. Hence, we concluded that the viral load measurements in this study were reliable.

5.4. Dried blood spots for viral load monitoring

In our study we found a good correlation between HIV-1 RNA viral load results in plasma and DBS from patients on ART. Although previous laboratory studies had reported similarly good results [74, 78-81], our study was the first to test this method in real life in rural Africa, the setting where it can be of most use.

Since the onset of our study, a number of other research groups have assessed the use of DBS for viral load quantification [123-138]; however, only two studies used samples from rural clinics in Africa [131, 133]. The study by Löfgren and colleagues is of particular interest, since it demonstrated the programmatic efficiency of DBS-based viral load quantification in a low-income country. DBS samples were collected in rural hospitals in Tanzania and sent by ordinary mail to the reference laboratory, and - in contrast to other studies - testing of samples was performed in the country [131]. Our study, together with others, indicate that virological monitoring in resource-limited settings now can be within reach.

However, there are some caveats. In DBS specimens with low viral loads (<3000 copies/mL) we observed a reduced sensitivity to detect HIV-1 RNA. Most other DBS studies have reported a detection limit around 1000 copies/mL, ranging from approximately 250 [80] to 5000 copies/mL [126]. For comparison, commercial plasma-based viral load assays can reliably quantify HIV-1 RNA down to 20-50 copies/mL. These ultrasensitive assays usually require an input volume of approximately 1 mL of plasma, equivalent to 2.5 mL of whole-blood. DBS consist of small volumes of whole-blood; each circle of a 903 filter paper card holds 75-80 µL of whole-blood when saturated [139]. The input volume to the nucleic acid amplification, therefore, is markedly reduced when DBS are used rather than plasma, and DBS-based HIV-1 RNA quantification can never achieve the same sensitivity as standard plasma-based methods. Nonetheless, further refinement of DBS-based methods could increase sensitivity to a satisfactory level.

We used the NucliSens EasyQ HIV-1 assay to measure viral load in DBS. This assay uses an isothermal transcription-based amplification system designed specifically for RNA detection [140-142]. In the absence of heat denaturation, double-stranded DNA can not participate in the amplification process. This is not relevant when plasma is used since plasma only contains cell-free RNA; however, when DBS are used for viral load quantification, proviral DNA from peripheral blood mononuclear cells can contribute in the amplification process. This might explain why some studies have reported an overestimation of viral load levels in DBS from patients with low-level viraemia (<5000 copies/mL in plasma). This phenomenon was observed in two studies using the Generic

HIV Charge Virale assay (Biocentric, Bandol, France) [135, 137], two studies using the m2000rt Real-Time HIV-1 assay (Abbott Laboratories, Abbott Park, IL, USA) [131, 132], and one study using the Cobas TaqMan Real-Time RT-PCR assay (Roche Diagnostics) [138]. All these assays are based on RT-PCR, which amplifies all nucleic acid material in the sample. Thus, a positive amplification result could be caused by either RNA or DNA or both. The contribution of proviral DNA to the viral load result was recently confirmed by Monleau and colleagues, using the Generic HIV Charge Virale assay: DBS treated with DNase yielded significantly lower viral loads than DBS tested in the absence of DNase [136]. However, the presence of HIV-1 DNA in a patient sample does not imply treatment failure, and joint amplification of RNA and DNA can lead to serious problems in the interpretation of a positive result. Studies using the NucliSens assay, on the contrary, did not report overestimation of HIV-1 RNA levels. NASBA technology, therefore, appears to be particularly suitable when DBS are used instead of cell-free plasma for HIV-1 RNA quantification.

A weakness of our study was that we used different viral load assays for plasma and DBS. Assay choice for plasma was based on availability in Tanzania, but we acknowledge that it would have been methodologically sounder to use the same assay for plasma and DBS. However, previous studies have found good agreement between the NucliSens and the Amplicor assay, although NucliSens on average gave slightly lower results than Amplicor [87, 143]. In fact, the scatter plots and regression analyses in these two studies, with an R^2 value of 0.75 and 0.7997 using plasma with both assays, were quite similar to our results ($R^2 = 0.75$). Amplicor gives viral load results as copies/mL

and NucliSens as IU/mL, but according to the manufacturer these are equivalent in version 1.2 of the NucliSens assay.

We used linear regression to compare HIV-1 RNA levels in plasma and DBS. Linear regression does not require normally distributed data; however, a prerequisite is that the dependent variable is continuous, which was fulfilled in our study [88]. In our data set, 33 plasma values and 61 DBS values were negative or below the lower limit of detection. For the purpose of analysis, these samples were reported at the cutoff value of the assay, which according to the manufacturer is 40 and 400 copies/mL for the TaqMan and Amplicor, respectively, when plasma is used. For the NucliSens assay using DBS, however, the lower limit of detection has not been established, and we chose 250 IU/mL as the cutoff value based on the lowest positive measurement in our study (280 IU/mL). This decision might have affected the regression line and the mean difference between the assays; however, the contribution of this was limited, and the strong correlation between plasma and DBS results remained after excluding results below the detection limit.

5.5. Dried blood spots for resistance testing

In our study we achieved a HIV-1 genotypic resistance result in 94% of DBS from patients with treatment failure, and there was good agreement with genotypes derived from plasma. Prior to the onset of our study there was only one publication on the use of DBS for HIV-1 resistance testing [82], but over the past three years several other research

groups have demonstrated the efficiency of this method [86, 130, 136, 144-151]. However, all other studies were carried out under standardized laboratory conditions, and we were the first to employ this method in a routine ART program in rural Africa.

We used an in-house method for genotyping, as did the majority of other studies to date. Commercial genotyping kits have several advantages over in-house assays, including quality-controlled reagents, standardized protocols and validated interpretation tools, which would facilitate routine use in high-throughput settings. However, the reduced sensitivity in samples with low viral load needs to be addressed before they can be recommended for clinical use. For example, Youngpairoj and colleagues, using the ViroSeq assay (Abbott Molecular), reported that only 8% of DBS could be genotyped when viral load was below 10,000 copies/mL, compared to 81% when viral load was above 10,000 copies/mL. When samples were re-tested with an in-house RT-nested PCR assay, however, 95% of samples were successfully genotyped [150]. Overall amplification success rates with in-house assays range from 83% to 100% [82, 86, 136, 144, 145, 149, 151], compared to 38.6% to 83.3% for the ViroSeq assay [130, 146, 148, 150] and 78.8% for the TruGene HIV-1 genotyping assay (Siemens Healthcare Diagnostics, Deerfield, IL, USA) [147]. Hence, in-house RT-nested PCR assays appear to be more efficient than commercial assays when DBS are used instead of plasma.

DBS consist of whole-blood, and cell-associated proviral DNA can be present in the nucleic acid sample. Some studies amplified DBS extracts in the absence of reverse transcriptase to assess whether proviral DNA contributed significantly to the

amplification product [82, 86, 144, 148, 151]. In these studies, the presence of proviral DNA was demonstrated in 33-80% of RT-PCR positive DBS samples. More recently, Monleau and colleagues reported that amplification efficiency from DBS was markedly reduced in samples treated with DNase, confirming that proviral DNA contributes significantly to the genotypes derived from DBS [136]. Of note, this latter study found that the relative contribution of DNA appeared to increase with duration of DBS storage, indicating that RNA might be degraded at a faster rate than DNA.

The interference of proviral DNA may vary according to treatment status and viral load level. In patients who fail treatment, more drug-resistance mutations tend to be detectable in plasma than in peripheral blood mononuclear cells, particularly when viral burden is low [152, 153]. On the contrary, in patients who interrupt treatment, proviral DNA can act as an archive of resistance mutations, and DBS might provide more information than plasma in such patients [153]. Most DBS studies to date, however, reported a high concordance between plasma-derived and DBS-derived sequences. Nucleotide similarity between the two sample types ranged from 98.1% [154] to 99.9% [144]. Drug-resistant mutations found in plasma were detected in 82% [86] to 100% [146, 147, 151] of the corresponding DBS specimens. Few additional mutations were detected in DBS, and those found were mainly as amino acid mixtures with the wild-type strain.

Two studies reported a significant discrepancy between genotypes derived from DBS and plasma. Buckton and colleagues observed several amino acid differences in paired plasma and DBS specimens [145]. However, the sample size was small (n=12), and only

two patients had clinically significant drug resistance; in both cases the plasma and DBS genotypes were discordant. Furthermore, Steegen and colleagues reported a lack of reproducibility for the detection of drug-resistant mutations in DBS, but this study specifically sequenced DNA and can not be compared directly to other studies which include an RT-PCR step [149]. By contrast, two studies reported a high degree of reproducibility from DBS specimens, with a mean nucleotide sequence concordance of 99.2% and 99.76% for the set of DBS replicates, using the commercial TruGene assay and an in-house assay, respectively [144, 147].

The main limitation of our study was the sample size. Ideally we should have included more than 36 plasma/DBS pairs for comparison, but the overall low rates of treatment failure in the program, although fortunate for the patients, limited the number of samples available for comparison.

Further, we were unable to assess the effect of different environmental conditions on the ability to genotype HIV-1 from DBS. However, other laboratory studies have assessed specific storage conditions in detail. Our aim was to assess how a DBS-based monitoring strategy would perform under field conditions, i.e. with storage at non-standardized ambient temperature for various lengths of time, which is closer to how the method would be applied in real life.

5.6. Could dried blood spots expand access to virological monitoring in resource-limited settings?

Our results could have direct implications for monitoring of HIV-infected individuals in resource-limited settings. The question is whether DBS are sufficiently reliable to be used for patient monitoring in routine clinical practice.

Viral load is usually measured in order to detect treatment failure and assess the need for second-line treatment. In resource-limited settings, where the selection of second-line antiretroviral drugs is scarce, the WHO recommends to conserve first-line treatment as long as viral load does not exceed 5000 copies/mL, because the risk of clinical progression is limited below this level [44, 45]. In our study, we found that DBS had reduced sensitivity to detect HIV-1 RNA when viral burden was low (<3000 copies/mL), which has also been observed by others [74, 126]. However, using a threshold of 5000 copies/mL to define major virological failure, as recommended by the WHO, DBS showed high sensitivity, specificity and predictive values. Thus, DBS appear to be sufficiently sensitive in combination with the WHO guidelines to decide who needs regimen switching in resource-limited settings.

With regard to resistance testing in routine clinical practice, this will become increasingly important as ART programs mature and more patients fail treatment. Currently, the WHO has no viable strategy to manage patients who fail second-line treatment [45]. In our study, DBS yielded a genotype in the vast majority of patients with treatment failure, and

there was a high concordance with plasma results. Most other studies have also reported good agreement with plasma, although certain theoretical caveats exist, such as the contribution of HIV-1 proviral DNA. The WHO, in collaboration with a network of international experts (HIVResNet), recently published a laboratory strategy for surveillance of transmitted HIV drug resistance (primary resistance in untreated individuals), where the use of DBS was recommended in resource-limited settings [155]. The WHO method using DBS has been successfully employed in surveys of transmitted drug resistance in Malawi, Tanzania, Iran and China [122, 156-158], and could be further adapted to routine use in clinical patient management. Each filter paper card contains 4-5 blood spots, of which 2 are needed for viral load quantification, and the remaining can be used for resistance testing in viraemic patients. Such a monitoring strategy would be fully feasible, but would demand an upgrading and funding of central laboratories.

5.7. Future research

Huge operational challenges still remain in the scale-up of ART. Still only 36% of those estimated to be in urgent need of ART receive it [46], and research should aim to identify even simpler delivery systems for large-scale treatment in remote and rural areas. Strategies to reduce the high early mortality in ART programs in Africa must be identified, including how to increase uptake in HIV testing services so that HIV-infected individuals who are unaware of their serostatus can get access to care and treatment before they develop severe immunodeficiency. Surveillance of drug resistance should be a priority globally to ensure that the empirical first- and second-line ART regimens recommended by the WHO are potent and efficient.

In the near future many programs will face the challenges of treatment failure and resistance even to second-line regimens. Viral load and resistance testing will inevitably become a pressing need in such settings. DBS can be a solution for viral load monitoring in resource-limited settings, but the method will have to be standardized and optimized for a high-throughput setting. Excision of blood spots and extraction of nucleic acids from DBS samples requires more hands-on time than automated systems designed for plasma samples, and carries the risk of cross-contamination. Although certain studies have explored simplified laboratory procedures, such as the use of a hand-held office hole-punch for excision of blood spots [159], there is a need for increased automation of the excision and extraction step to allow for mass testing in high-prevalence settings. Furthermore, standardization of viral load assays for DBS usage is required, and it should be ascertained whether there are significant differences between PCR-based and NASBA-based assays in patients with low-level viraemia.

With regard to drug resistance testing on DBS, most studies have utilized in-house assays for genotyping from DBS, and there is a need for standardization and quality control of the protocols used by different laboratories. The WHO has developed recommendations for the use of DBS in surveillance of transmitted HIV drug resistance, and these can serve as a guide for the use of DBS in clinical patient management [155]. However, further research is warranted to explore the interference of proviral DNA according to treatment status and viral load level, as some studies have indicated possible differences between

plasma and DBS in early treatment failure and in patients who interrupt treatment [152, 153].

The ideal solution for virological monitoring in resource-limited settings would be a simple, stable, robust and affordable point-of-care assay. This would remove the difficulties related to transport of samples and reporting back results, and facilitate immediate treatment decisions at the testing site. Rather than to force-fit high-tech solutions into resource-constrained health systems, research should focus on invention of low-tech point-of-care assays, both for HIV-1 RNA viral load quantification and drug resistance testing. A semi-quantitative dipstick HIV-1 RNA assay is under development by a research group at Cambridge University, but is not yet commercially available [160]. Other efforts to develop point-of-care assays are still far from materializing into available products. Thus, for the coming years, DBS appear to be the only viable option for viral load quantification and resistance testing in settings with limited laboratory capacity.

6. Concluding remarks

This thesis highlights that favorable long-term virological efficacy of ART can be achieved in rural Africa, and that drug resistance appears to develop at the same rate as in high-income countries. However, the high early mortality in our study underscores the need to identify HIV-infected individuals and start ART earlier in the course of their illness. We identified anemia, malnutrition and thrombocytopenia as predictors of mortality, and found that a prognostic model based on hemoglobin level was particularly useful for initial risk assessment.

In our study the use of DBS was a feasible and reliable option for viral load and resistance testing in rural Tanzania. It is my hope that our work will contribute to increased access to virological monitoring in resource-limited settings, and it is encouraging to notice that the newest version of the NucliSens viral load assay comes with a standardized protocol for DBS.

Unfortunately, at the time I submit this thesis there are darks clouds in the horizon. Many of the large institutional donors are now reducing their contributions to ART programs, based on short-sighted financial considerations. At the same time, mathematical models have suggested that universal HIV testing with immediate ART can reverse the pandemic and nearly eliminate HIV by 2050 [161]. It is my sincere belief that a sustained commitment to the scale-up of ART, combined with continued focus on research, will be the only way to combat the HIV pandemic.

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Appendix

Name_____Surveillance No._____
170306/JNB

HAYDOM LUTHERAN HOSPITAL - HIV TREATMENT UNIT NCTP-ID no.:_____

HISTORY SHEET – INCLUSION DATE…../…../20… Date of filling form:…../…../20…

To be used for all patients. Laboratory results from the inclusion should be entered on the space for baseline. For patients included before January 2005 only subsequent laboratory results after Jan. 2005 to be entered. Staging first column (A) to be filled in according to baseline examination.

Age_____ and/or Date of Birth…../…../…… Sex *M*□ *F*□

Examiner_____.

Address *Village*_____*Ward*_____*Division*_____*District*_____

Referred from *TB*□ *MED*□ *SUR*□ *MAT*□ *PED*□ *OPD*□ *RCH*□ *VCT*□ *Other*□_____

Previous HIV-test *Y*□ *N*□ *Date……/……/…….. HIV+*□ *HIV-*□ *Lab.records seen Y*□ *N*□

First HIV+ve confirmatory test Blood..…./…../20..…

Second HIV+ve confirmatory test Blood..…./…../20..… .HAVACOP No._____

Travel: Walking□ *Bicycle*□ *Bus/car*□ Travelling distance:_____km

Marital status *Si*□ *M*□ *Co*□ *Sep*□ *D*□ *W*□

Ten Cell Leader_____

Guardian/Contact person: Name_____Relationship:_____.

Does the contact person know about your HIV status: *Y*□ *N*□

Can we discuss your HIV with the contact person*: Y*□ *N*□

Religion *Christian*□ *Muslim*□ *Traditional*□ *Hindu*□ *Other*□

Ethnic -tribe/race: *Datooga*□ *Iraqw*□ *Nyiramba*□ *Nyaturu*□ *Nyisanzu*□ *Sukuma*□ *Hadzabe*□

Other tribe/race □ *Specify:*_____

Can you read? *Y*□ *N*□ Can you write? *Y*□ *N*□

*Primary school (1-7y)*___*yrs Sec. school (1-6y)*___*yrs Other training*___*yrs*

Occupation: *Farm r* □ *Business* □ *Governm inst* □ *Health inst* □ *Religious inst* □ *Driver* □ *Housewife* □ *Other* □. _____

Patners occupation (if applicable): Farmer □ *Business* □ *Governm inst* □ *Health inst* □ *Religious inst* □ *Driver* □ *Housewife* □ *Other* □._____

Socioeconomic situation: *Selfprovided.*□

Dependent on: *Spouse* □ *Parents.*□ *Childr..*□ *Other family* □

Crops □ *Other* □ *Specify:*_____

Sexual partner(s): *No. present(last 6 months)?:* ____*No. lifetime?:* _____

For women – *Pregnant? Y* □ _____*weeks N* □ *Number of children: total:*_____*alive:*_____

Years of birth for living children_____

Breastfeeding Y □ _____*months N* □

Any other known PLWH/A in the family(alive):_____Number:_____

Other members of your family dying from HIV/AIDS: _____Number:_____

Name_____ Surveillance No._____
170306/JNB

MEDICAL HISTORY

Any Tuberculosis or chronic cough in the family_____.

History of TB in patient: Never had TB □

- Previous TB Pulmonary No □ Y□ *AFB+*□ *AFB÷*□ *Unknown* □ Date(s)_____

Extrapulmonary□ Location_____Date(s)_____

- Within last year TB: No □ Y□ Pulmonary *AFB+*□ *AFB÷*□ *Unknown*□ Date(s)_____.

Extrapulmonary□ Location:_____Date(s)_____

Are you on treatment for tuberculosis *N*□ *Y*□ *Start date:*___/___/___

Intensive phase Y□ *Continuation phase Y*□

Blood transfusion.(year) *Y*□ _____*N*□ Allergy *Y*□ *N*□_____.

Hospitalisations (month)/ year/ diagnosis_____.

Other diseases/ description_____

CURRENT POSSIBLY HIV RELATED SYMPTOMS (specify on provided line)

Yes No (Y= yes, N= no)

□ □ Fever

□ □ Chronic fever and weakness > 1 month *Duration*_____

□ □ Weight loss. If yes: > 10 %? Y □ ____kg/_____ months <10 %? Y □ If not known estimate!

□ □ Night Sweats _____

□ □ Cough. If yes duration?_____Productive?_____

□ □ Shortness of breath_____

□ □ Diarrhoea for less than 1 month. Duration:_____

□ □ Chronic intermittent diarrhoea > 1 month. If yes duration?_____

□ □ Sore throat. If yes duration:_____

□ □ Oral thrush (candida)_____

□ □ Kaposi in mouth:_____

□ □ Pain (specify site)_____

□ □ Odynophagia (retrosternal pain on swallowing)_____

□ □ Nausea/ vomiting (duration)_____

□ □ Headache (duration)_____

□ □ Recurrent (probable HSV-) sores (duration) Lips_____Genitals_____

□ □ Herpes Zoster Previous (year)_____Presently_____

□ □ Encephalopathy/ Neurological symptoms: _____

□ □ Recurrent RTIs/ severe infections (specify)_____

□ □ Genitourinary symptoms (specify)_____

□ □ Oedema_____

□ □ Bedridden during normal daytime due to sickness <50%□ >50% □_____

□ □ Other (Skin and nail changes etc.)_____

Name_____Surveillance No._____
170306/JNB

MEDICATION

Have you ever got ARV medication? *Y*□ *N* □ If yes when:_____where_____

Present ARV medication (instituted from others)? *Y*□ *N*□

Are you on drugs for opportunistic infection prophylaxis / treatment: *Y*□ *N*□

If yes: □ Cotrimoxazole. Specify:_____

□ INH prophylaxis. Specify:_____

□ INH as treatment □ Rifampicin □Pyrazinamide □ Ethambutol

□ Other medication taken by the patient. Specify:_____

_____

EXAMINATION

Current weight_____Best/normal weight_____ Height_____Temp_____

BP_____HR_____Head circumference (children)_____cm Oedema *N*□ +□ ++□ +++□

General state *Well* □ *Wasted* □ *Bedridden* □ *Unconscious* □

Lymphadenopathy (>1cm) *N* □ *Neck R* □ *L* □ *Axillae R* □ *L* □ *Inguinal R* □ *L* □

Mouth *N* □ *Thrush* □ *Kaposi* □ *Ulce r*□ *Hairy Leucopl* □ *Gingivitis* □

Eyes *N* □ *Pale* □ *Yellow* □ *Sunken* □

Skin *N* □ *Rash* □ *Kaposi* □ *Other tumor /abnormalities* □ *specify* _____

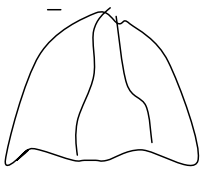
Nails *N* □ *Fungal infection* □ *Other* □ *Specify*_____

Heart *N* □ *Abnormal* □

Lungs *N* □ *Abnormal* □Chest X-ray *N* □

Cavity □ *Other abnormality*

Specify _____



Abdomen *N* □ *Abnormal* □ *Hepatosplenomegaly* □

Extremities/ groin *N* □ *Abnormal* □ Genitals *N* □ *Abnormal* □ *Not examined* □

Signs of new central nervous system abnormalities (Cognitive deterioration) *Y* □ *N* □

*If yes specify:*_____

Peripheral neuropathy *Y* □ *N* □

*If yes specify:*_____

Other symptoms or findings. Specify:_____

_____

_____

_____

(N = no, normal or negative)

Name_____ Surveillance No._____
170306/JNB

STAGING (F= First examination, A= Accumulated) (Check guidelines for more exact definitions)

ADULTS AND ADOLESCENTS (≥15 years)

F A F: First examination A:accumulated

WHO Stage I

□ □ 1. Asymptomatic
□ □ 2. Persistent generalized lymphadenopathy

WHO stage II

□ □ 3. Weight loss, <10 % of presumed body weight
□ □ 4. Minor mucocutaneous manifestations (papular pruritic eruption, seborrhoeic dermatitis, angular cheilitis, recurrant oral ulcers)
□ □ 5. Herpes zoster
□ □ 6. Recurrent URTI (≥2 in 6 months)
□ □ 33. Fungal nail infections

WHO Stage III

□ □ 7. Weight loss, >10 % of body weight
□ □ 8. Unexplained chronic diarrhoea, >1 month
□ □ 9. Unexplained persistent fever (intermittent or constant), >1 month
□ □ 10. Oral candidiasis (thrush)
□ □ 11. Oral hairy leukoplakia
□ □ 12. Pulmonary tuberculosis (current)
□ □ 34. Tuberculous lymphadenitis
□ □ 13. Severe bacterial infections (i.e. pneumonia, pyomyositis)
□ □ 35. Acute necrotizing ulcerative gingivitis or periodontitis
□ □ 30. Unexplained anemia (<8), neutropenia (<0.5) or chronic thrombocytopenia (<50)

WHO Stage IV

□ □ 14. HIV wasting syndrome (= combination of 7 and 8 or 7 and 9)
□ □ 15. Pneomocystis Carinii Pneumonia
□ □ 32. Recurrent (≥2 in 6 months) severe or radiologically confirmed bacterial pneumonia
□ □ 16. Toxoplasmosis of the brain
□ □ 17. Chronic cryptosporidiosis or isosporiasis with diarrhoea > 1month
□ □ 18. Cryptococcosis, extrapulmonary
□ □ 19. Cytomegalovirus disease (other than liver, spleen or lymph node)
□ □ 20. HSV infection, mucocutaneous > 1 month, or visceral any duration
□ □ 21. Progressive multifocal leukoencephalopathy
□ □ 22. Any disseminated endemic mycosis (i.e. histoplasmosis, coccidioidomycosis)
□ □ 23. Candidiasis of the oesophagus, trachea, bronchi or lungs
□ □ 24. Atypical mycobacteriosis, disseminated
□ □ 25. Recurrent non-typhoid Salmonella septicaemia
□ □ 26. Extrapulmonary tuberculosis (excluding lymphnode tbc)
□ □ 27. Lymphoma
□ □ 28. Kaposi's sarcoma
□ □ 29. HIV encephalopathy
□ □ 36. Invasive cervical carcinoma
□ □ 37. Symptomatic HIV-associated nephropathy or cardiomyopathy
□ □ 38. Atypical disseminated leishmaniasis

WHO STAGE (Date)

Adults	Children
I_____	**P I:**_____
II_____	**P II:**_____
III_____	**P III:**_____
IV_____	**P IV:**_____

CHILDREN

F A

WHO Stage P I

□ □ P1. Asymptomatic
□ □ P2. Persistent generalized lymphadenopathy

WHO Stage P II

□ □ P3. Unexplained persistent hepatosplenomegaly
□ □ P4. Mucocutaneous manifestations (papular pruritic eruption, lineal gingival erythema, recurrent oral ulcers)
□ □ P5. Unexplained persistent parotid enlargement
□ □ P6. Extenisve HPV or mollusc. contagiosum
□ □ P7. Recurrent or chronic URTI (≥2 in 6 months)
□ □ P8. Herpes zoster
□ □ P9. Fungal nail infections

WHO Stage P III

□ □ P10. Unexplained moderate malnutrition
□ □ P11. Unexplained chronic diarrhoea, >14 days
□ □ P12. Unexplained persistent fever, >1 month
□ □ P13. Oral candidiasis (outside neonatal period)
□ □ P14. Oral hairy leukoplakia
□ □ P18. Recurrent (≥2 in 6 months) severe or radiologically confirmed bacterial pneumonia
□ □ P33. Unexplained anemia (<8), neutropenia (<0.5) or chronic thrombocytopenia (<50)
□ □ P34. Pulmonary tuberculosis
□ □ P36. Lymph node tuberculosis
□ □ P37. Lymphoid interstitial pneumonitis
□ □ P38. Chronic HIV-associated lung disease
□ □ P39. Acute necrotizing ulcerative gingivitis or periodontitis

WHO Stage P IV

□ □ P25. Unexplained severe wasting, stunting or severe malnutrion
□ □ P15. Extrapulmonary tuberculosis
□ □ P16. Other disseminated mycobacteriosis
□ □ P17. Candidiasis of the oesophagus, trachea, bronchi or lungs
□ □ P19. Cryptococcal meningitis
□ □ P20. CNS toxoplasmosis
□ □ P21. HIV encephalopathy
□ □ P22. Cerebral lymphoma
□ □ P23. Kaposi's sarcoma
□ □ P24. Recurrent septicaemia or meningitis
□ □ P26. Pneumocystis carini pneumonia
□ □ P27. Recurrent severe bacterial infections (≥2 in 6 months), excluding pneumonia
□ □ P28. Chronic orolabial or cutaneous HSV (>1 month), or visceral any site
□ □ P35. Cryptosporodiosis or isosporiasis with diarrhoea >1 month
□ □ P40. Cytomegalovirus infection (age >1 month)
□ □ P41. Disseminated endemic mycosis
□ □ P42. Progressive multifocal leukoencephalopathy
□ □ P43. Symptomatic HIV-associated nephropathy or cardiomyopathy

In infants < 18 mos (HIV antibody positive): two or more of the following:

□ □ P29. Oral candidiasis
□ □ P30. Severe pneumonia
□ □ P32. Severe sepsis

I

Paper I

Predictors of mortality in HIV-infected patients starting antiretroviral therapy in a rural hospital in Tanzania

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Predictors of mortality in HIV-infected patients starting antiretroviral therapy in a rural hospital in Tanzania

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Abstract

Background: Studies of antiretroviral therapy (ART) programs in Africa have shown high initial mortality. Factors contributing to this high mortality are poorly described. The aim of the present study was to assess mortality and to identify predictors of mortality in HIV-infected patients starting ART in a rural hospital in Tanzania.

Methods: This was a cohort study of 320 treatment-naïve adults who started ART between October 2003 and November 2006. Reliable CD4 cell counts were not available, thus ART initiation was based on clinical criteria in accordance with WHO and Tanzanian guidelines. Kaplan-Meier models were used to estimate mortality and Cox proportional hazards models to identify predictors of mortality.

Results: Patients were followed for a median of 10.9 months (IQR 2.9–19.5). Overall, 95 patients died, among whom 59 died within 3 months of starting ART. Estimated mortality was 19.2, 29.0 and 40.7% at 3, 12 and 36 months, respectively. Independent predictors of mortality were severe anemia (hemoglobin <8 g/dL; adjusted hazard ratio [AHR] 9.20; 95% CI 2.05–41.3), moderate anemia (hemoglobin 8–9.9 g/dL; AHR 7.50; 95% CI 1.77–31.9), thrombocytopenia (platelet count $<150 \times 10^9$/L; AHR 2.30; 95% CI 1.33–3.99) and severe malnutrition (body mass index <16 kg/m^2; AHR 2.12; 95% CI 1.06–4.24). Estimated one year mortality was 55.2% in patients with severe anemia, compared to 3.7% in patients without anemia ($P < 0.001$).

Conclusion: Mortality was found to be high, with the majority of deaths occurring within 3 months of starting ART. Anemia, thrombocytopenia and severe malnutrition were strong independent predictors of mortality. A prognostic model based on hemoglobin level appears to be a useful tool for initial risk assessment in resource-limited settings.

Background

The introduction of highly active antiretroviral therapy in 1996 dramatically improved the prognosis for HIV-infected patients in the industrialized world [1,2]. Until recently, however, access to treatment has been severely limited in developing countries, where the majority of people with HIV/AIDS live [3]. In 2002, the World Health Organization (WHO) issued guidelines for scaling up antiretroviral therapy (ART) in resource-limited settings, followed by revisions in 2003 and 2006 advocating earlier initiation of treatment [4-6]. By December 2006, two million people in low- and middle-income countries were receiving ART, but this was still only 28% of those estimated to be in urgent need of it [7].

Few studies have examined the effect of ART in rural Africa, and experiences from Europe and North America are not necessarily applicable to such settings. However, early reports from ART programs in resource-limited settings have been promising, with virological efficacy comparable to industrialized countries [3]. Nevertheless, mortality has been high, particularly the first months after initiating ART [8-15], and factors contributing to this high mortality are poorly understood.

A better knowledge of prognostic factors would allow closer follow-up and more targeted interventions in high-risk patients, thus reducing excess mortality. The aim of the present study was to assess mortality and to identify predictors of mortality in HIV-infected patients starting ART in a rural African hospital.

Methods

Study setting and participants

Tanzania is a low-income country in East Africa with 38.3 million inhabitants and estimated adult HIV prevalence at 6.5% [7]. Life expectancy at birth is 46.5 years, which is estimated to be ten years lower than it would have been without the HIV epidemic [16]. Haydom Lutheran Hospital is a 400-bed hospital in Manyara region owned by the Evangelical Lutheran Church of Tanzania. It is the main health care provider to a rural population of about 260 000 people, and available services include a modern radiology department with ultrasonography and computer tomography, a fairly well equipped laboratory with microscopy, bacteriology and biochemistry, as well as standard surgical and obstetrical services. According to a recent population-based survey, adult HIV prevalence in the area is 1.8% [17]. In 2002, the hospital launched a comprehensive HIV prevention and intervention program with emphasis on voluntary counseling and testing (VCT) through outreach services and antenatal clinics. An HIV Care and Treatment Centre was established adjacent to the hospital, and from October 2003 ART was provided free of charge to eligible HIV-infected patients. Most of the patients enrolled were detected through VCT services in the villages or were hospitalized patients tested on clinical suspicion. Clinical officers, under supervision of a physician, were responsible for medical follow-up of patients. On-site training was provided by HIV specialists from collaborating institutions in Norway. All patients received pre-treatment counselling, and peer-support groups were set up in the major villages. A community home-based care network was established to follow-up adherence and trace missing patients.

Patients were considered eligible for ART if they were in WHO stage IV irrespective of CD4 cell count, WHO stage III with CD4 ≤ 350 cells/μL, or had CD4 ≤ 200 cells/μL regardless of clinical stage, in accordance with WHO and Tanzanian guidelines [5,18]. However, since CD4 cell counts measured by manual techniques were observed to be unreliable, ART initiation was based solely on clinical criteria (WHO stage III and IV) in most patients. In addition, ART was offered to HIV-infected pregnant and lactating women to prevent vertical transmission.

The present study is a prospective, observational cohort study of treatment-naïve patients aged 15 years or older who started ART in Haydom Lutheran Hospital between October 3, 2003, and November 5, 2006. Women who were pregnant at the time of ART initiation were excluded from the study, as were lactating mothers in WHO stage I or II, who started ART exclusively to prevent vertical transmission. Follow-up data was collected through May 5, 2007. Patients gave written consent to participate in the study. Ethical approval was obtained from the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania and Regional Committee for Medical Research Ethics in Norway.

Treatment, monitoring and endpoints

First-line treatment comprised stavudine (d4T) or zidovudine (ZDV), combined with lamivudine (3TC), and either nevirapine (NVP) or efavirenz (EFV). Regimen choice was subject to availability, with use of a generic fixed-dose combination of d4T, 3TC and NVP whenever possible. Second-line treatment in case of treatment failure was not available until December 2006. Patients with CD4 ≤ 200 cells/μL or WHO stage III or IV disease were given co-trimoxazole prophylaxis 960 mg thrice weekly or 480 mg daily. After the initial 2 weeks of daily drug administration, antiretroviral drugs were dispensed on a monthly basis.

A standardized form was used for the baseline evaluation, which included socio-demographic information, medical history, physical examination, and laboratory investigations. Clinical staging was performed using the 2003 revision of the WHO clinical staging system [5]. Routine

clinical follow-up was scheduled every 3 months. HIV infection was established using 2 different rapid antibody tests. Standard hematology was measured using Sysmex KX-21 Hematology Analyzer (Sysmex Corp., Kobe, Japan).

The most recent laboratory results before starting ART were generally used as baseline values. In a minority of patients who lacked pre-treatment laboratory tests, however, results obtained within one month of ART initiation were used. If two values were obtained within a month, the mean was employed. Body mass index (BMI, weight in kilograms divided by height in meters squared) was used to assess nutritional status. Body weight was measured at each clinic visit using the same manual scale, and height was measured using a stadiometer mounted on the scale. Established cutoff values for BMI were used [19]: normal (BMI ≥ 18.5 kg/m^2), mild malnutrition (BMI 17–18.4 kg/m^2), moderate malnutrition (BMI 16–16.9 kg/m^2), and severe malnutrition (BMI < 16 kg/m^2). Anemia was defined as a hemoglobin level of <12 g/dL for women and <13 g/dL for men [20], and was classified as mild (hemoglobin 10–11.9 g/dL for women and 10–12.9 g/dL for men), moderate (hemoglobin 8–9.9 g/dL) or severe (hemoglobin < 8 g/dL). Lymphopenia was defined as a total lymphocyte count (TLC) of <1.2 × 10^9/L [4], and we employed an additional cutpoint at 0.6 × 10^9/L to assess severe lymphopenia. Thrombocytopenia was defined as platelet count <150 × 10^9/L [21].

The main endpoint in our study was death from all causes. Deaths were registered from hospital records or reported through home visitors. Other outcomes were also recorded, including patients who self-stopped treatment, were transferred to another health facility or were lost to follow-up. Patients who missed appointments for more than 3 months and could not be traced by the home visitor, were regarded lost to follow-up.

Statistical analysis

Patients were excluded from the study if sex, age or WHO stage was not recorded. Date of death was registered by home visitors; however, in 7 patients with only month and year recorded we used the 1st of that month, and in 2 patients with unknown death date we used the last follow-up visit. For subjects who self-stopped treatment, were transferred out or were lost to follow-up, the date of their last follow-up visit was used as the censoring date. Finally, individuals alive and on ART were censored at May 5, 2007.

Kaplan-Meier models were used to estimate survival after ART initiation, and log rank tests to compare survival curves. Cox proportional hazards models were used to identify independent predictors of mortality and calculate hazard ratios. Multicollinearity was excluded using Spearman's correlation coefficient with a cutoff at 0.7. We performed univariable Cox regression analysis for the following baseline variables: sex, age, tribe, religion, education level, ART start year, WHO stage, BMI, hemoglobin, TLC, platelet count, hepatitis B, syphilis and active tuberculosis (TB). CD4 cell counts were omitted since the results were observed to be inaccurate. Baseline variables significant at $P < 0.05$ level in univariable analysis were included in the final multivariable model. We used SPSS version 14.0 software (SPSS Inc., Chicago, IL, USA) to analyze the data. All tests were two-sided and level of significance was set at $P < 0.05$.

Results

Baseline characteristics

Of 779 patients enrolled into HIV care between October 3, 2003, and November 5, 2006, 320 treatment-naïve non-pregnant adults who started ART were included in the present study. The cohort profile is presented in figure 1. Among 334 adults who had not started ART at censoring, 123 (36.8%) were lost to follow-up, 90 (26.9%) did not meet clinical criteria for starting ART, 56 (16.8%) died before ART initiation, 27 (8.1%) were transferred to another health facility, and the remaining 38 (11.4%) were still waiting to start treatment.

Patients on ART were followed for a median of 10.9 months (interquartile range 2.9–19.6). Summary statistics of baseline characteristics are given in table 1. Of the 320 patients included, 223 (69.7%) were women and median age was 35 years (interquartile range 30–43). There were 104 patients (32.5%) who started ART in the initial years 2003–04, 117 (36.6%) started in 2005, and 99 (30.9%) in 2006. Initial ART regimen was d4T/3TC/NVP in 168 patients (52.5%), d4T/3TC/EFV in 58 (18.1%), ZDV/3TC/NVP in 53 (16.6%), ZDV/3TC/EFV in 24 (7.5%), ZDV/3TC/tenofovir in one (0.3%) and missing in 15 patients (4.7%). Seventy-three patients received anti-TB treatment at inclusion or started after inclusion. Mean BMI was 17.6 kg/m^2 (standard deviation [SD] 3.1), mean hemoglobin 10.1 g/dL (SD 2.1), mean TLC 1.4 × 10^9/L (SD 0.8) and mean platelet count 266 × 10^9/L (SD 131).

At ART initiation, 210 patients (65.6%) had clinical AIDS (WHO stage IV). For comparison, 401 (51.5%) of 779 had clinical AIDS at enrollment into the HIV program. The most common WHO stage IV conditions among patients who started ART were: wasting syndrome (89.0%), oesophageal candidiasis (13.3%), extrapulmonary TB (5.2%) and Kaposi's sarcoma (4.8%).

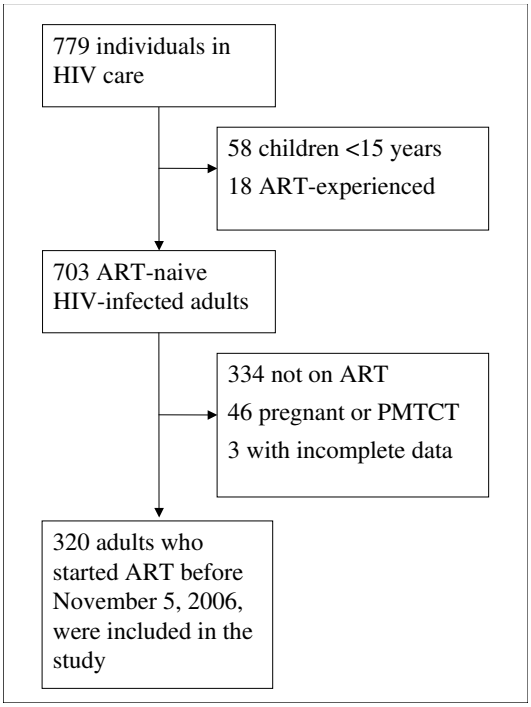

Figure 1
Profile of the study cohort, Haydom Lutheran Hospital, Tanzania (October 2003–November 2006).

Survival analysis

Overall, 95 patients (29.7%) died during the follow-up period, among whom 59 died within 3 months of starting ART. Thirty-five patients (10.9%) were transferred to another health facility, 31 (9.7%) were lost to follow-up and 7 (2.2%) self-stopped treatment. Estimated mortality was 19.2, 24.5, 29.0, 35.2 and 40.7% at 3, 6, 12, 24 and 36 months, respectively.

In univariable analysis male sex, ART start year, WHO stage IV, severe malnutrition, anemia, lymphopenia and thrombocytopenia were all associated with progression to death. No such associations were found for age, tribe, religion, education level, hepatitis B, syphilis or active TB. As described in table 1, certain baseline values were missing in 29 patients; hence, there were 291 patients in the final Cox model. In multivariable analysis significant predictors of mortality were severe and moderate anemia, thrombocytopenia and severe malnutrition (Table 2). The hazard of death was significantly reduced in those starting ART in calendar year 2006 compared with the initial period 2003–04.

Mortality increased with decreasing hemoglobin. Estimated one year mortality was 3.7% in patients without anemia, 20.0% in mild anemia, 37.6% in moderate anemia and 55.2% in severe anemia (log rank test, $P < 0.001$, Figure 2). The majority of deaths occurred early, and the corresponding 3 months mortality was 3.7, 8.1, 26.9 and 40.4%, respectively (log rank test, $P < 0.001$). A similar trend was observed with decreasing BMI. Estimated one year mortality was 13.7% in patients with normal nutritional status, 21.0% in mild to moderate malnutrition, and 46.8% in severe malnutrition (log rank test, $P < 0.001$, Figure 3).

Discussion

Mortality was high in this cohort, and most of the deaths occurred within 3 months of starting ART. Severe and moderate anemia, thrombocytopenia and severe malnutrition were found to be independent predictors of mor-

Table 1: Baseline characteristics and associated mortality among 320 HIV-infected patients starting ART in Tanzania

Characteristic	Number of patients	Number of Deaths
Age (years)		
15–24	26	7 (26.9%)
25–34	129	39 (30.2%)
35–44	95	30 (31.6%)
≥ 45	70	19 (27.1%)
Sex		
Male	97	38 (39.2%)
Female	223	57 (25.6%)
Clinical stage		
WHO stage I–II	12	1 (8.3%)
WHO stage III	98	18 (18.4%)
WHO stage IV	210	76 (36.2%)
BMI (kg/m^2)[a]		
<16	98	46 (46.9%)
16–18.4	105	23 (21.9%)
≥ 18.5	93	14 (15.1%)
Hemoglobin (g/dL)[b]		
<8	49	27 (55.1%)
8–9.9	108	43 (39.8%)
10–11.9 (10–12.9 for men)	104	21 (20.2%)
≥ 12 (≥ 13 for men)	55	2 (3.6%)
TLC (× 10^9/L)[c]		
<0.6	30	18 (60.0%)
0.6–1.1	116	32 (27.6%)
≥ 1.2	166	42 (25.3%)
Platelet count (× 10^9/L)[d]		
<150	52	24 (46.2%)
≥ 150	261	66 (25.3%)

[a]24 values missing (n = 296). [b]4 values missing (n = 316). [c]8 values missing (n = 312). [d]7 values missing (n = 313).
WHO, World Health Organization; BMI, body mass index; TLC, total lymphocyte count.

tality. The high early mortality observed in our study is in line with other similar studies from resource-limited settings [8-15]. Causes of death were not investigated in the present study; however, in a study from South Africa wasting syndrome, TB, acute bacterial infections, malignancies and immune reconstitution disease were the major causes of death [14]. In our cohort more than half of the patients had clinical AIDS at enrollment into HIV care, and other African ART programs have also reported high rates of advanced disease [8-12,14,15]. Stigma and delay in seeking health care, lack of voluntary testing and counseling services, and health system delays in referral and ART initiation are possible explanations. Thus, priority must be given to identify HIV-infected individuals and start treatment earlier in the course of their illness, before they develop severe opportunistic infections.

Anemia was a strong predictor of mortality in our study. Patients with severe anemia had nearly 15 times higher risk of dying during the first year on ART compared to those with a normal hemoglobin level. Several studies from Europe and North America have shown that anemia is an independent predictor of mortality in patients on ART, even after controlling for CD4 cell count and viral load [22-24]. Recently, studies from developing countries have found the same association [9,13]. Indeed, in the largest African cohort study published to date, severe anemia (hemoglobin <8 g/dL) was the strongest independent predictor of mortality in 16 198 patients receiving ART in Zambia [13].

It is uncertain whether the association between anemia and mortality is causal or whether anemia is rather a marker of progressive HIV disease. It is known that the incidence of anemia increases with progression of HIV infection [23]. Furthermore, anemia can be a feature of certain opportunistic diseases, like disseminated mycobacterial infection and parvovirus B19 [25]. Several other etiologic factors may be involved in the development of HIV-associated anemia, including micronutrient deficiencies, immunological myelosuppression, impaired erythropoietin production and blood loss from intestinal opportunistic disease [25]. The role of iron supplementation is controversial, as some reports have suggested adverse effects of iron excess in HIV-infected individuals in industrialized countries [26,27]. On the contrary,

Table 2: Hazard ratios of mortality according to baseline variables in HIV-infected patients starting ART in Tanzania

	Unadjusted		Adjusted[a]	
Baseline variables	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Gender (male vs. female)	1.73 (1.15–2.61)	0.009	1.60 (1.00–2.57)	0.053
WHO stage (IV vs. I–III)	2.71 (1.64–4.49)	<0.001	1.46 (0.81–2.65)	0.210
ART start year (vs. 2003–04)				
2005	0.55 (0.35–0.87)	0.010	0.64 (0.38–1.08)	0.091
2006	0.30 (0.17–0.56)	<0.001	0.40 (0.19–0.83)	0.014
BMI (vs. ≥ 18.5 kg/m^2)				
<16	4.17 (2.29–7.60)	<0.001	2.12 (1.06–4.24)	0.034
16–18.4	1.60 (0.82–3.10)	0.168	1.27 (0.62–2.61)	0.516
Hemoglobin (vs. ≥ 12 g/dL for women and ≥ 13 for men)				
<8	22.7 (5.40–95.7)	<0.001	9.20 (2.05–41.3)	0.004
8–9.9	13.5 (3.28–55.9)	<0.001	7.50 (1.77–31.9)	0.006
10–11.9 (10–12.9 for men)	6.21 (1.46–26.5)	0.014	4.03 (0.93–17.5)	0.063
TLC (vs. ≥ 1.2 × 10^9/L)				
<0.6	3.58 (2.05–6.24)	<0.001	1.72 (0.87–3.39)	0.117
0.6–1.1	1.10 (0.69–1.74)	0.699	0.79 (0.48–1.32)	0.371
Platelet count (<150 vs. ≥ 150 × 10^9/L)	2.23 (1.40–3.57)	0.001	2.30 (1.33–3.99)	0.003

[a]Cox proportional hazards model adjusted for all variables listed in the table.
HR, hazard ratio; CI, confidence interval; ART, antiretroviral therapy; WHO, World Health Organization; BMI, body mass index; TLC, total lymphocyte count.

recovery from anemia after erythropoietin treatment has been associated with improved survival [23,24], but high costs limit its use in poor countries. More recently, ART has been shown to significantly reduce HIV-associated anemia in developed countries [28,29]; however, this has not yet been investigated in rural Africa. Further studies are needed to explore possible interventions against HIV-associated anemia in resource-limited settings, including the role of iron supplementation.

Malnutrition was another strong, independent predictor of mortality in our study. Estimated one year mortality was nearly 50% among patients with severe malnutrition. Previously, studies from industrialized countries have shown that malnutrition in HIV infection is associated with morbidity and mortality, even after the introduction of highly active antiretroviral therapy in the late 1990s [30-32]. More recently, studies from developing countries have found that malnutrition is an independent predictor of mortality in patients starting ART [8,12,13,33]. However, it is not clear whether targeted therapy for malnutrition will result in improved survival [34]. Studies of nutritional interventions in HIV patients are urgently needed in developing countries, where malnutrition is often a result of poverty and food insecurity.

We found a reduced risk of death in patients starting ART in later calendar years compared with the initial period 2003–04. A possible explanation is that many patients with severe AIDS were included in the initial period, as this was the first clinic to offer ART in the area. However, since the risk reduction persisted after controlling for clinical stage, we believe that it may also be attributed to improved skills among local staff managing HIV patients. The decline in mortality over time supports our experience that non-physician clinicians can be trained to follow-up and treat HIV-infected patients.

To our knowledge, thrombocytopenia has never previously been shown to predict mortality in African patients on ART, although a few studies from North America have described an increased risk of disease progression and death [35,36]. Further research is needed to confirm our findings. WHO stage IV was not significantly associated with mortality in our study, in contrast to previous reports [1,8,11-14]. However, the comparison group was almost entirely composed of WHO stage III patients, which would weaken the statistical effect of WHO stage IV on mortality. Furthermore, the accuracy of clinical staging is probably quite variable in rural Africa. It is interesting that simple and more objective indicators identified in the present study appear to have a better predictive ability than clinical stage.

A prognostic model based on hemoglobin level had a strong predictive power in our study, separating the patients into low, low intermediate, high intermediate and high risk groups (Figure 2). Previously, similar survival curves for hemoglobin levels have been reported in European HIV patients, although anemia occurred less frequently [22]. Hemoglobin is a simple and inexpensive laboratory test, which can be performed even in rural,

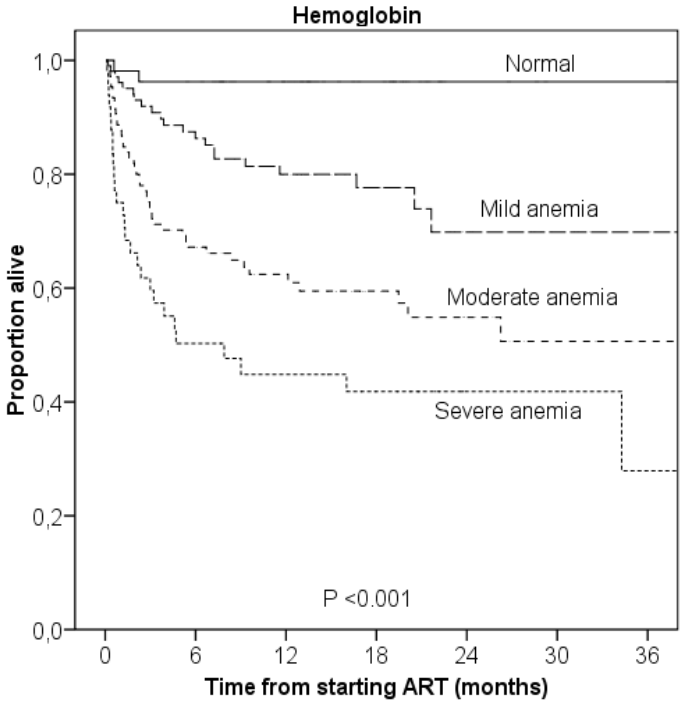

Figure 2
Kaplan-Meier survival curves according to baseline hemoglobin. Normal: >12 g/dL (>13 g/dL for men); mild anemia: 10–11.9 g/dL (10–12.9 g/dL for men); moderate anemia: 8–9.9 g/dL; severe anemia: <8 g/dL.

basic clinics. We believe it can be used as a simple and practical tool for initial risk assessment in the absence of CD4 cell count and viral load. Such early prognostic information would allow a more targeted search for opportunistic infections and closer follow-up in high-risk individuals, thus reducing excess mortality. Although the exact mortality figures from the present study can not necessarily be applied to other populations, we believe the concept of using hemoglobin level to identify patients with a poor prognosis can be used elsewhere. This simple prognostic model should be tested out in other African settings to assess its generalizability.

There are some weaknesses of our study. First, mortality might be underestimated, since patients lost to follow-up probably include individuals dying at home without being reported. Although the proportion of patients lost to follow-up in the present study (9.7%) was comparable to other African studies [12,13], data quality would be improved with better cohort retention. Second, the results might be affected by selection bias towards patients with more severe disease, since the study was conducted in a hospital setting. Third, some patients measured baseline hemoglobin shortly after ART initiation, which might have led to an overestimation of the prevalence of anemia in patients with a ZDV-based regimen. However, post-ART hemoglobin was only employed in a small number of patients, and it is unlikely that this has introduced any systematic bias into the study. Fourth, it is known that the generalizability of a prognostic system can be impaired if important independent predictors are left out [37]. We lacked reliable CD4 cell counts and viral loads, which are established predictors of morbidity and mortality in patients on ART [1]. However, our results strongly suggest that simple and available measurements can be useful alternative prognostic markers.

The main strength of our study is that it was carried out in a rural African hospital with use of national staff and inclusion of all eligible patients. Most other African ART

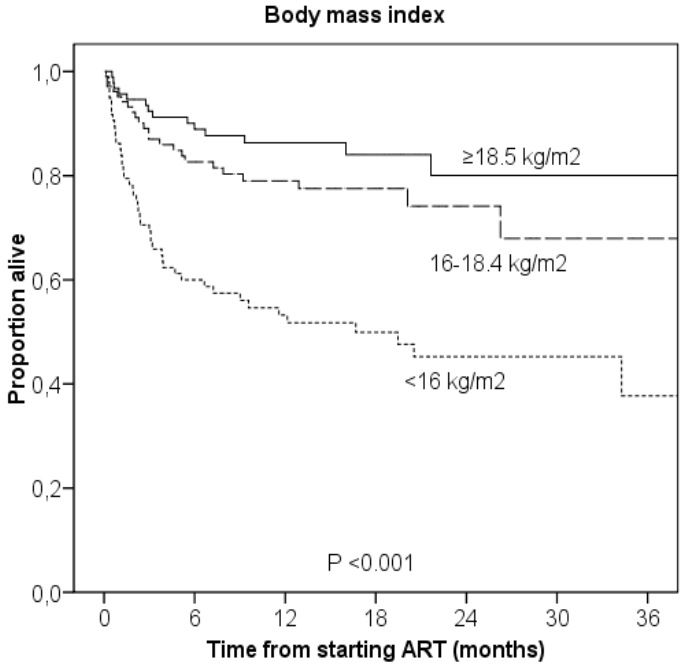

Figure 3
Kaplan-Meier survival curves according to baseline body mass index.

studies have been performed in urban areas [9-11,13-15], in research settings with strict inclusion and exclusion criteria [38], or with support from an international non-governmental organization [8,10,12]. We believe that our results better reflect the reality in a rural hospital in sub-Saharan Africa, and thus may be applicable to other similar settings.

Conclusion

We found high mortality among patients starting ART in this rural Tanzanian hospital, with the majority of deaths occurring within 3 months of ART initiation. Many patients enrolled with advanced immunodeficiency, and priority should be given to identify HIV-infected individuals and start ART earlier in the course of their illness. Anemia, thrombocytopenia and severe malnutrition were strong independent predictors of mortality. A simple prognostic model based on hemoglobin level appears to be a useful tool for initial risk assessment in resource-limited settings.

Competing interests

The author(s) declares that they have no competing interests.

Authors' contributions

AJ analyzed the data and drafted the manuscript. EN and BJN collected the data. LS performed the statistical analysis and helped to draft the manuscript. MIM participated in the conception of the study. HEA participated in the data collection and design of the study. SGG and JNB conceived the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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Virological efficacy and emergence of drug resistance in adults on antiretroviral treatment in rural Tanzania

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II

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Virological efficacy and emergence of drug resistance in adults on antiretroviral treatment in rural Tanzania

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Abstract

Background: Virological response to antiretroviral treatment (ART) in rural Africa is poorly described. We examined virological efficacy and emergence of drug resistance in adults receiving first-line ART for up to 4 years in rural Tanzania.

Methods: Haydom Lutheran Hospital has provided ART to HIV-infected patients since October 2003. A combination of stavudine or zidovudine with lamivudine and either nevirapine or efavirenz is the standard first-line regimen. Nested in a longitudinal cohort study of patients consecutively starting ART, we carried out a cross-sectional virological efficacy survey between November 2007 and June 2008. HIV viral load was measured in all adults who had completed at least 6 months first-line ART, and genotypic resistance was determined in patients with viral load >1000 copies/mL.

Results: Virological response was measured in 212 patients, of whom 158 (74.5%) were women, and median age was 35 years (interquartile range [IQR] 29–43). Median follow-up time was 22.3 months (IQR 14.0–29.9). Virological suppression, defined as <400 copies/mL, was observed in 187 patients (88.2%). Overall, prevalence of ≥1 clinically significant resistance mutation was 3.9, 8.4, 16.7 and 12.5% in patients receiving ART for 1, 2, 3 and 4 years, respectively. Among those successfully genotyped, the most frequent mutations were M184I/V (64%), conferring resistance to lamivudine, and K103N (27%), Y181C (27%) and G190A (27%), conferring resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs), whereas 23% had thymidine analogue mutations (TAMs), associated with cross-resistance to all nucleoside reverse transcriptase inhibitors (NRTIs). Dual-class resistance, i.e. resistance to both NRTIs and NNRTIs, was found in 64%.

Conclusion: Virological suppression rates were good up to 4 years after initiating ART in a rural Tanzanian hospital. However, drug resistance increased with time, and dual-class resistance was common, raising concerns about exhaustion of future antiretroviral drug options. This study might provide a useful forecast of drug resistance and demand for second-line antiretroviral drugs in rural Africa in the coming years.

Background

Access to antiretroviral treatment (ART) of HIV/AIDS has increased substantially over the past few years throughout the developing world. Lower prices of antiretroviral drugs combined with political determination have given rise to one of the greatest public health operations of our time, spearheaded by World Health Organization (WHO), Joint United Nations Programme on HIV/AIDS (UNAIDS) and international non-governmental organizations (NGOs). By December 2007, three million people were receiving ART in low- and middle-income countries, but still this was only 31% of those estimated to be in need of it [1].

ART programs in developing countries are now moving from early pioneer projects to a sustained effort. Inevitably, the long-term challenges of providing ART will become increasingly evident, including late drug toxicities, treatment failure and emergence of drug resistance [2-4]. Indeed, some have argued that scaling up ART in Africa could create widespread drug resistance [5,6]. Early reports, however, have documented good adherence to therapy [7] and short-term virological efficacy comparable to industrialized countries [8].

Although several studies on ART efficacy in Africa have been published, the majority have been carried out in larger cities [9-11], often with NGO support [10,12], and usually with short follow-up time [9,10,12]. However, the majority of Africans reside in rural areas [13], and little is known about the long-term effects of ART in such settings. The key to long-term benefit of ART is sustained suppression of viral replication and avoidance of resistance [14-16]. Our aim was to assess virological efficacy and emergence of drug resistance in HIV-infected patients up to 4 years after starting first-line ART in a rural Tanzanian hospital.

Methods

Study setting, participants and treatment

Tanzania is a low-income country with an estimated HIV prevalence of 6.2% [1]. The National AIDS Control Program started to scale up antiretroviral treatment from 2005, and by December 2007, 135,696 people were receiving ART [1]. Haydom Lutheran Hospital is a 400-bed hospital in Manyara region owned by the Evangelical Lutheran Church of Tanzania. It is the main health care provider to a rural population of about 260,000 people. In 2002, the hospital launched a comprehensive HIV prevention and intervention program, which has previously been described in detail [17]. In brief, free treatment and care has been offered to all HIV-infected patients since October 2003, including free drugs and in-patient care. Clinical officers have been trained by experienced HIV physicians to treat and follow-up patients. The HIV program in Haydom is now integrated in the National AIDS Control Program.

All patients were assessed with a standardized evaluation form at enrolment, where demographic data, medical history, clinical findings and laboratory investigations were recorded. ART was initiated in accordance with WHO's recommendations [18-20]: WHO stage 4 irrespective of CD4 cell count, WHO stage 3 with CD4 ≤350 cells/μL, or CD4 ≤200 cells/μL with any WHO stage. However, reliable CD4 cell counts were not available until September 2006; thus, most patients started ART based on clinical criteria only (WHO stage 3 and 4). In addition, triple-drug combination ART, and not single-dose nevirapine, was offered to HIV-infected pregnant and lactating women, from pregnancy week 20 till cessation of breast-feeding, irrespective of WHO stage and CD4 cell count, to prevent mother-to-child transmission (PMTCT).

First-line treatment was stavudine or zidovudine, combined with lamivudine, and either nevirapine or efavirenz. A generic fixed-dose combination of stavudine, lamivudine and nevirapine was preferred whenever possible. Patients with CD4 ≤200 cells/μL or WHO stage 3 or 4 disease were given co-trimoxazole prophylaxis 960 mg thrice weekly. Second-line treatment was available from December 2006 and comprised lopinavir/ritonavir, didanosine and abacavir. Criteria for switching to second-line ART was virological failure as recommended by WHO (i.e. >10,000 copies/mL) [20]; however, viral load was not measured routinely, and only selected patients with high clinical suspicion of failure were tested.

Nested in a longitudinal cohort study of patients consecutively starting ART, we carried out a cross-sectional virological efficacy survey between November 15, 2007 and June 6, 2008. All adults (≥15 years) who had received first-line ART for more than 6 months were considered eligible. Patients were included regardless of previous adherence or treatment interruptions. However, those who had stopped ART for ≥1 month and not re-started at the time of the survey were classified as "stopped treatment" and excluded. Furthermore, those who had already switched to second-line ART were excluded since genotypic resistance results prior to the switch were unavailable. Ethical approval was granted by National Institute for Medical Research in Tanzania and Regional Committee for Medical Research Ethics in Norway, and all patients gave written consent to participate in the study.

Laboratory investigations

Standard laboratory investigations at baseline included: Full blood cell count, erythrocyte sedimentation rate, liver function tests, creatinine, blood sugar, hepatitis B surface antigen and syphilis serology. Patients who started ART were followed up with laboratory investigations every three months. Hematology was measured using the Sysmex KX-21 Hematology Analyzer (Sysmex Corp., Kobe, Japan). CD4 cell counts were available from September

2006 using the FACSCount flow cytometer (Becton Dickinson, San Jose, California, USA).

Plasma specimens for virological analyses were stored at -20°C until shipment to the reference laboratory. Manufacturer's instructions were followed with regard to sample collection and transport. HIV viral load was measured at Muhimbili National Hospital, Dar es Salaam, Tanzania, using the Cobas TaqMan 48 Analyzer (Roche Diagnostics, Branchburg, New Jersey, USA) with a lower detection limit at 40 copies/mL; however, due to equipment breakdown, one third of the samples were analysed with the Cobas Amplicor HIV-1 Monitor v1.5 (Roche Diagnostics, Branchburg, New Jersey, USA) with a detection limit at 400 copies/mL. All specimens with viral load >1000 copies/mL were sent to Ulleval University Hospital, Oslo, Norway, for genotypic resistance testing. The ViroSeq HIV-1 Genotyping System (Abbott Molecular, De Plains, Illinois, USA) was used to determine HIV-1 subtype and mutations in the protease and reverse transcriptase genes. Only drug resistance mutations listed in the Spring 2008 update from the International AIDS Society were considered [21]. Resistance profiles to antiretroviral drugs were interpreted according to the Stanford University HIV Drug Resistance Database (HIVdb Program, http://hivdb.stanford.edu).

Statistical analysis

The main outcomes of interest were on-treatment virological suppression and clinically significant genotypic resistance. Virological suppression was defined as HIV viral load <400 copies/mL, since this was the detection limit of the least sensitive assay used in this study. Clinically significant genotypic resistance was defined as HIV viral load >1000 copies/mL and presence of ≥1 drug resistance mutation listed in the Spring 2008 update from the International AIDS Society [21]. Duration of ART was rounded off to the nearest full year (1, 2, 3 or 4 years) when presenting prevalence of virological suppression and drug resistance. Logistic regression was used to study associations between baseline characteristics and emergence of drug resistance. Univariable analysis was performed for the following variables: Sex, age, WHO stage, initial ART regimen, duration of ART, body mass index, hemoglobin level and total lymphocyte count. CD4 cell counts were excluded because of too few observations. Variables with $P < 0.1$ in univariable analyses were advanced into a multivariable regression analysis, using the forward stepwise (Wald) method to avoid overcorrection. Multicollinearity was excluded using Spearman's correlation coefficient with a cutoff at 0.7. Data were analysed with SPSS version 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA), except 95% confidence intervals (CI) for proportions which were calculated with NCSS version 2007 (NCSS, Kaysville, Utah, USA). All tests were two-sided and level of significance was set at $P < 0.05$.

Results

Baseline characteristics

Out of 549 adults who enrolled in the HIV program and started ART, 126 patients (23.0%) died, of whom 76 died within 3 months of starting ART. Seventy-nine patients (14.4%) were transferred to another health facility, 46 (8.4%) were lost to follow-up, whereas 27 patients (4.9%) self-stopped treatment after receiving ART for a median of 11.3 months. Fifty patients were not eligible for the virological survey because they had taken ART for less than 6 months, and 3 because they were on second-line ART. Among the remaining 218 patients who were selected for the survey, plasma was obtained from 212 of them. Six patients failed to participate due to: Temporary travel to another area ($n = 3$), error in specimen preparation ($n = 1$) or unknown ($n = 2$). The study profile is presented in figure 1.

Median follow-up time among 212 patients included in this study was 22.3 months (interquartile range [IQR] 14.0–29.9). Median age was 35 years (IQR 29–43), and 158 patients (74.5%) were women. At the time of ART initiation, 110 (52.1%) had clinical AIDS (WHO stage 4). The most common AIDS defining events were: Wasting syndrome (87.3%), oesophageal candidiasis (10.9%),

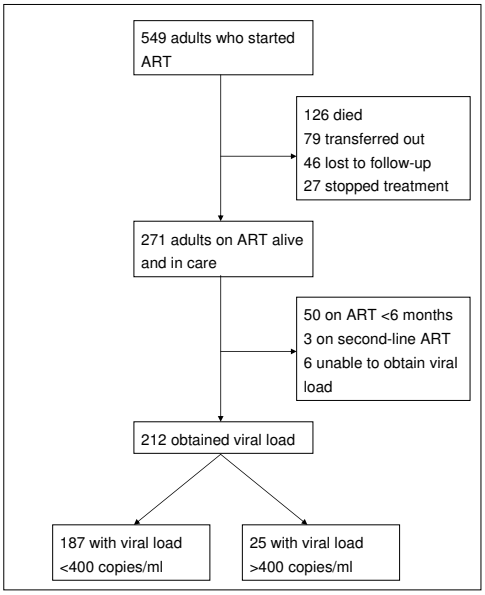

Figure 1
Profile of the study cohort, Haydom Lutheran Hospital, Tanzania, 2003–08.

extrapulmonary tuberculosis (4.5%) and Kaposi's sarcoma (4.5%). Initial ART regimen was stavudine/lamivudine/nevirapine in 122 patients (57.5%), stavudine/lamivudine/efavirenz in 39 (18.4%), zidovudine/lamivudine/nevirapine in 45 (21.2%), and zidovudine/lamivudine/efavirenz in 6 (2.8%). Among 66 patients with a baseline CD4 measurement, median CD4 cell count was 118 cells/μL (IQR 51–189).

Virological results

Overall, 187 patients (88.2%; 95% CI 83.1–92.2) had suppressed viraemia (<400 copies/mL). Two patients (0.9%) had 400–1000 copies/mL, 14 (6.6%) had 1000–10,000 copies/mL, 5 (2.4%) had 10,000–100,000 copies/mL, and 4 (1.9%) had >100,000 copies/mL. The proportion of patients (95% CI) with suppressed viraemia after 1, 2, 3 and 4 years was 94.8% (87.2–98.6), 88.0% (79.0–94.1), 75.0% (57.8–87.9) and 87.5% (61.7–98.4), respectively (figure 2). The small number of patients who received ART for 3 and 4 years (n = 36 and n = 16, respectively) gave rise to wide confidence intervals for those groups.

Genotyping was successful in 22 of 23 samples with viral load >1000 copies/mL. HIV-1 subtypes were A (n = 3), C (n = 7), D (n = 8) and CRF01_AE (n = 2), whereas 2 were inconclusive (different subtypes in the protease and reverse transcriptase genes). Among those successfully genotyped, 18 patients (82%) harboured at least one clinically relevant resistance mutation in the reverse transcriptase gene (table 1). The most frequent mutations were M184I/V (n = 14; 64%), conferring resistance to lamivudine, and K103N (n = 6; 27%), Y181C (n = 6; 27%) and G190A (n = 6; 27%), conferring resistance to non-nucleoside reverse transcriptase inhibitors (NNRTIs). Thymidine analogue mutations (TAMs), associated with cross-resistance to all nucleoside reverse transcriptase inhibitors (NRTIs), were found in 5 patients (23%), of whom 1 had ≥3 TAMs. Fourteen patients (64%) had dual-class resistance, i.e. resistance to both NRTIs and NNRTIs. None had clinically relevant mutations in the protease gene.

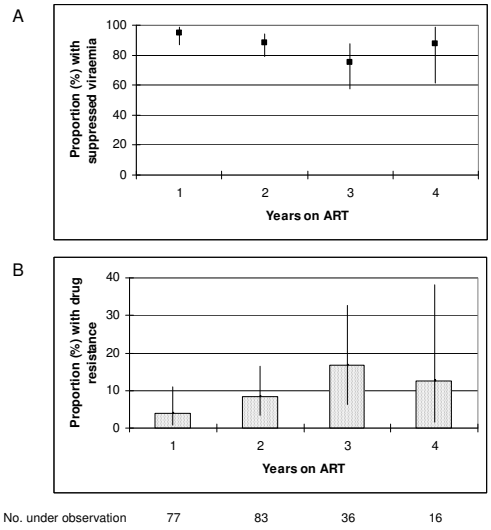

Figure 2
Proportion of patients on ART with: A) suppressed viraemia (<400 copies/mL), and B) ≥1 clinically significant resistance mutation. Vertical lines indicate 95% confidence interval.

Hence, in total, 18 of 212 patients (8.5%; 95% CI 5.1–13.1) harboured drug resistance by use of a standard genotyping assay. The prevalence (95% CI) of any clinically significant drug resistance after 1, 2, 3 and 4 years was 3.9% (0.8–11.0), 8.4% (3.5–16.6), 16.7% (6.4–32.8) and 12.5% (1.6–38.3), respectively (figure 2). Dual-class resistance was observed in 3.9% (0.8–11.0), 6.0% (2.0–13.5), 13.9% (4.7–29.5) and 6.3% (0.2–30.2), respectively. Again, the small number of patients on ART for 3 and 4 years gave rise to wide confidence intervals.

Predictors of drug resistance

In univariable logistic regression analysis only duration of ART was significantly associated with emergence of drug resistance (≥3 years on ART; odds ratio [OR] 4.49; 95% CI 1.13–17.8; P = 0.033). Anemia (hemoglobin <10 g/dL; OR 2.84; 95% CI 0.97–8.32; P = 0.058) and lymphopenia (total lymphocyte count <1.2 × 10^9/L; OR 2.91; 95% CI 0.99–8.53; P = 0.052) were borderline significant. No associations were found for age, sex, clinical stage, body mass index or initial ART regimen. In multivariable analysis where duration of ART, anemia and lymphopenia were included using the forward stepwise method, only duration of ART remained in the final model, with the same odds ratio and P-value as above (table 2).

Two patients with low-level viraemia (400–1000 copies/mL) and one patient whose genotyping failed were assumed not to harbour resistance in our study. To assess whether this assumption might have biased our results, we conducted a sensitivity analysis where these patients were classified as resistant. In the resulting multivariable model, both duration of ART (≥3 years on ART; OR 6.47; 95% CI 1.28–32.6; P = 0.024) and lymphopenia (total lymphocyte count <1.2 × 10^9/L; OR 4.24; 95% CI 1.48–12.2; P = 0.007) were significantly associated with resistance. Hence, the effect of lymphopenia might have been

Table 1: Genotypic resistance results in 23 patients on ART with virological failure (HIV-1 RNA >1000 copies/mL)

ID	Sex	Age	ART regimen: initial (current[a])	Months on ART	Subtype	Viral load	Protease mutations	Reverse transcriptase mutations
34	M	24	ZDV/3TC/NVP (ZDV/3TC/EFV)	42.1	C	434,131	M36I, L63P, H69K, I93L	K103N
84	M	30	d4T/3TC/EFV (d4T/3TC/NVP)	49.1	D	8690	I13V, L33V, M36I, I64V	K103N, M184V
224	M	32	d4T/3TC/NVP	34.7	C	1349	M36I, L63P, H69K, I93L	
240	M	43	d4T/3TC/EFV (d4T/3TC/NVP)	35.5	CRF01_AE	81,691	I13V, M36I, L63P, H69K	D67N, K70R, K103N, V179T, M184V, K219Q
275	F	41	d4T/3TC/NVP	32.3	D	477,518	I13V, K20R, M36V, L63P, I64V, I93L	G190A
282	F	32	d4T/3TC/NVP	32.4	A	2621	L10I, I13V, M36I, H69K	M184V, Y188C
307	F	35	d4T/3TC/NVP (d4T/3TC/EFV)	31.7	*Failed*	2301	*Failed*	*Failed*
321	F	34	d4T/3TC/NVP	30.4	D	1504	I13V, K20R, M36I, I62V, I64V	Y181C, M184I
366	F	48	d4T/3TC/NVP	28.3	C	3000	M36I, H69K, I93L	K103N, V179T, M184V
401	F	44	d4T/3TC/NVP	28.6	C	2290	M36I, D60E, H69K, I93L	G190A
402	F	40	d4T/3TC/NVP (d4T/3TC/EFV)	28.3	?[b]	20,500	L10I, I13V, G16E, M36I, H69K	K101P, M184V, G190A, T215F
410	F	45	d4T/3TC/NVP	36.9	D	5990	I13V, L63P, I64V, V77I	K101E, M184V, G190A
473	F	35	d4T/3TC/NVP	27.3	D	3965	I13V, I64V, V77I	M41L, V75I, Y181C, M184V
476	F	19	ZDV/3TC/NVP (d4T/3TC/NVP)	26.5	D	1980	I64V	G190A
516	F	24	d4T/3TC/EFV	24.0	C	419,979	M36I, L63P, H69K, I93L	
554	F	28	ZDV/3TC/NVP (d4T/3TC/NVP)	24.0	C	1886	K20R, M36I, H69K	
583	F	30	d4T/3TC/NVP	21.1	CRF01_AE	1432	L10V, G16E, M36IV, H69K	K103N, Y181C, M184V
611	F	33	d4T/3TC/NVP	17.8	C	15,600	M36I, I62V, L63P, H69K, V82I, I93L	K65R, V75I, V108I, Y181C, M184V, L210W
643	F	37	d4T/3TC/NVP	31.3	D	2400	K20R, M36I, I62V, I64M	K70R, Y181C, M184V
749	F	32	d4T/3TC/EFV (d4T/3TC/NVP)	19.0	?[c]	35,400	I13V, M36I, H69K	K103N, M184V
752	F	35	d4T/3TC/NVP	14.5	A	3101	L10I, M36I, H69K	Y181C, M184V
785	F	26	d4T/3TC/NVP (d4T/3TC/EFV)	14.6	D	3,683,117	I13V, M36I, D60E, I64V	
982	F	15	d4T/3TC/NVP	8.5	A	76,700	I13V, M36I, L63P, H69K, V82I	V179T, M184V, G190A

[a]Only specified if different from initial regimen.[b]CRF_01AE in the protease and A in the reverse transcriptase gene. [c]A in the protease and CRF_01AE in the reverse transcriptase gene.
ART, antiretroviral treatment; d4T, stavudine; 3TC, lamivudine; NVP, nevirapine; EFV, efavirenz; ZDV, zidovudine.

underestimated by misclassification bias in the main analysis, whereas duration of ART was a strong and significant predictor of resistance in both analyses.

Discussion

Virological suppression rates were good up to 4 years after starting ART in a rural Tanzanian hospital. These results are in keeping with early reports from resource-limited settings, where short-term virological efficacy rates were as good as those reported from Europe and North America [8]. We show that suppression of viraemia can be sustained for several years even in rural Africa, where logistical support is challenging and patients often live in poverty. However, like in many other African ART programs the attrition rate was high, and strategies to reduce early mortality and other program losses need to be identified [11,12,22,23].

Experiences elsewhere have shown that poorly managed HIV programs can give rise to widespread drug resistance [24]. A number of factors may have contributed to the sustained virological efficacy of the ART program in the present study. First, all treatment and care for HIV-infected patients was provided free of charge, which has previously been shown to improve treatment efficacy [8]. Second, all patients had three days of adherence counselling with a nurse prior to starting ART. Third, a close collaboration between the clinical staff and a network of community home-based carers ensured follow-up of patients in their villages. Fourth, regular educational peer-

Table 2: Predictors of drug resistance in 212 HIV-infected adults on ART in rural Tanzania

	Univariable[a]		Multivariable[b]	
Predictor variables	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Duration of ART				
1 year	1		1	
2 years	2.27 (0.57–9.12)	0.247	2.27 (0.57–9.12)	0.247
≥ 3 years	4.49 (1.13–17.8)	0.033	4.49 (1.13–17.8)	0.033
Hemoglobin				
≥ 10 g/dL	1			
<10 g/dL	2.84 (0.97–8.32)	0.058	NS	
Total lymphocyte count				
≥ 1.2 × 10^9/L	1			
<1.2 × 10^9/L	2.91 (0.99–8.53)	0.052	NS	

[a] No significant associations were found for age, sex, clinical stage, body mass index or initial ART regimen.
[b]Stepwise multivariable logistic regression using a forward selection procedure. NS denotes that the variable did not meet the significance level criterion (*P* <0.05) for inclusion in the final model.
OR, odds ratio; CI, confidence interval; ART, antiretroviral therapy.

support meetings contributed to reduce the stigma and isolation many patients experience after receiving an HIV diagnosis. Fifth, antiretroviral drug supply continuity was uninterrupted from the beginning of the program. And finally, visiting HIV physicians focused on capacity building of local clinical officers, with emphasis on common curable opportunistic infections, such as tuberculosis, candidiasis, cryptococcal meningitis and cerebral toxoplasmosis.

Overall, emergence of drug resistance was relatively uncommon; only 8.5% harboured clinically significant resistance mutations. Although the proportion of patients with drug resistance was low, however, in a high-prevalence country like Tanzania the absolute number of individuals in need of second-line ART can rapidly become substantial. If our results were extrapolated to Tanzania as of December 2007 [1], then 11,500 patients would harbour drug resistance and be in need of second-line ART. Such an extrapolation is not necessarily valid, but it illustrates the magnitude of the problem that drug resistance can inflict on national ART programs. The number of individuals receiving ART has increased 20-fold over the past 4 years in sub-Saharan Africa [1], but access to second-line antiretroviral drugs is still limited in many developing countries due to higher costs and lack of fixed-dose combinations. Our study underscores the growing global need for affordable and convenient second-line antiretroviral regimens.

The prevalence of drug resistance increased with time and reached approximately 15% after 3–4 years on ART. Most previous studies on ART in Africa have focused on early treatment efficacy, showing good virological results with a limited observation time [10,12,25]. Only a few studies have assessed long-term (>2 years) emergence of drug resistance in sub-Saharan Africa. An early study from Senegal showed that 12.5% had one or more drug resistance mutations after a median of 30 months on ART [26], whereas a recent study from Côte d'Ivoire found 22% resistance after a median of 37 months on ART [27]. These results should not be used as an argument against HIV treatment in Africa; in fact, the results are comparable to a recent study from Canada, where 20% developed resistance after 30 months on the ART regimen most widely used in resource-limited settings (stavudine/lamivudine/nevirapine) [28]. Thus, emergence of drug resistance appears to occur at a similar rate in Africa as in a Western setting.

Of concern, among 18 patients with drug resistance mutations, 14 harboured dual-class resistance. All 14 had a combination of M184I/V, conferring resistance to lamivudine, with one or more of K103N, Y181C and G190A, conferring resistance to NNRTIs. Five of these patients also had thymidine analogue mutations (TAMs), associated with cross-resistance to all NRTIs. In 3 patients the standard second-line regimen in Tanzania would not be adequate, i.e. would not introduce at least 2 fully active drugs, which is the recommended strategy in treatment failure [29]. Other studies from low- and middle-income countries, using the same first-line treatment, have found a similar pattern. In a recent study from Angola, 65% of patients with virological failure had dual-class resistance [30]. Furthermore, a study from Thailand found that second-line treatment options, in the absence of newer antiretroviral drugs, were limited for 48% of patients failing their initial regimen [31]. Expanding access to newer antiretroviral drugs, including new HIV drug classes, should be a priority in the global efforts to control HIV/AIDS.

It has been shown that in the presence of a failing ART regimen, resistance mutations accumulate, jeopardizing future treatment options [32]. Early detection of treatment failure rely on viral load measurements, a standard component of ART programs in resource-rich countries [29]. In the present program, like in most resource-limited settings, viral load was not measured routinely, and treatment failure had to be assessed by clinical signs and CD4 cell counts. However, clinical signs and CD4 decline, as recommended by WHO to detect treatment failure in the absence of viral loads, have poor sensitivity and specificity, and result in frequent misclassifications [33,34]. Hence, there is an urgent need for a simple, affordable viral load assay adapted for use in basic, tropical environments, so that treatment failure can be detected before multiple mutations occur.

In our study only duration of ART was significantly associated with emergence of drug resistance. Baseline anemia and lymphopenia were borderline significant in univariable analysis, and our sample size might have been too small to reveal a true association. Other studies have found that low CD4 cell count and high viral load at baseline increase the risk of drug resistance [35]; however, these measurements were not available in our study.

There were some weaknesses of this study. First, virological efficacy could only be assessed in patients who were alive and in care. A high early mortality accounted for most of the program loss, which probably reflected advanced immunodeficiency at enrolment rather than treatment failure [17]. Many patients were transferred out when the National AIDS Control Program started scaling up ART in other villages, but it is unlikely that this introduced any systematic bias. Among patients who stopped treatment (4.9%) or were lost to follow-up (8.4%), there was probably a proportion who either died or developed drug resistance. Our study must be considered an "on-treatment" analysis, and virological suppression rates and resistance estimates in an "intention-to-treat" analysis would have been poorer. Another limitation of this study was the lack of longitudinal viral load and resistance results. A cross-sectional virological survey may be more influenced by random biological variations and laboratory artefacts, being derived from a single time point. Also, we can not ascertain whether drug resistance mutations existed prior to initiation of ART, which was recently observed in rural South Africa [36], or developed during treatment. However, Haydom Lutheran Hospital was the first ART provider in the area, and single-dose nevirapine was not used for PMTCT, so it is unlikely that there was any significant primary resistance. Furthermore, this study was limited by lack of adherence data, which is considered the most important predictor of resistance [35]; however, adherence estimates would not have altered our conclusions. Finally, this was a hospital based study and probably there was a selection bias towards more advanced immunodeficiency at baseline, which has previously been shown to increase the risk of drug resistance [35]. On the other hand, late presentation has been observed in many African ART programs [10-12,25,37,38], and we believe our findings can be representative of other similar settings.

Conclusion

We found good virological suppression rates up to 4 years after initiating ART in a rural hospital in Tanzania. These results suggest that ART can be safely scaled up in rural Africa with similar long-term virological efficacy rates as those reported for industrialized countries. However, prevalence of drug resistance increased with time, and dual-class resistance was common, raising concerns about exhaustion of future antiretroviral drug options. Earlier detection of treatment failure and timely switch to second-line ART could reduce accumulation of drug resistance, underscoring the growing need for virological monitoring in resource-limited settings. This study might provide a useful forecast of drug resistance and demand for second-line antiretroviral drugs in rural Africa in the coming years.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AJ analyzed the data and drafted the manuscript. EN collected the data. MJK and MH-P were responsible for the laboratory analyses. MIM participated in the conception of the study. SLK participated in the data collection and coordination of the study. SGG and JNB conceived the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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Paper III

Dried blood spots perform well in viral load monitoring of patients who receive antiretroviral treatment in rural Tanzania

Clinical Infectious Diseases 2009, 49: 976-81

III

Paper IV

HIV type-1 drug resistance testing on dried blood spots is feasible and reliable in patients who fail antiretroviral therapy in rural Tanzania

Antiviral Therapy 2010, 15: 1003-1009

IV